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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

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CONTENTS

No. 1 (FEBRUARY)

PAGE

OBITUARY NOTICE: Adrian John Brown 1

I. Estimation of Carbon Dioxide, Oxygen and Combustible Gases by Krogh's Method of Micro-Analysis. By H. O. SCHMIT-JENSEN. (With two figures) 4

II. On the Deterioration of Cotton on Wet Storage. By N. FLEMING and A. C. THAYSEN. (With Plate I) 25

III. Adsorptive Stratification in Gels, III. By S. C. BRADFORD. (With Plate II) 29

IV. On the Association of Antitoxins with the Proteins of Immunised Horse Serum. By A. HOMER 42

V. The Guanidine Content of Faeces in Idiopathic Tetany. By J. S. SHARPE 46

VI. On the Colours of two Sea Anemones, *Actinia equina* and *Anemonia sulcata*. Part I. Environmental. By R. ELMHIRST. Part II. Chemical. By J. S. SHARPE. (With Plates III, IV) 48

VII. The Penetration of Electrolytes into Gels. I: The Penetration of Sodium Chloride into Gels of Agar-agar containing Silver Nitrate. By W. STILES. (With four figures) 58

VIII. Note on the Use of Butyl Alcohol as a Solvent for Anthocyanins. By O. ROSENHEIM 73

No. 2 (APRIL)

IX. An Investigation of the Methods Employed for Cooking Vegetables, with special reference to the losses incurred. Part II. Green Vegetables. By H. MASTERS and P. GARBUTT 75

X. On the Theory of Gels. II: The Crystallisation of Gelatin. By S. C. BRADFORD. (With one figure) 91

XI. A Note on the Effect of Purgation on the Creatinine Content of Urine. By D. BURNS 94

XII. The Preparation of Sörensen's Phosphate Solutions when the Pure Salts are not available. By C. J. MARTIN 98

XIII. The Influence of Reaction on Colour Changes in Tyrosine Solutions. By E. C. V. VENN. (With one figure) 99

XIV. Barger's Microscopical Method of Determining Molecular Weights. Part I. The Principle of the Method with Reference to the Molecular and Ionic Attraction of Solute for Solvent. By K. YAMAKAMI 103

XV. The Formation of Ferrous Sulphide in Eggs during Cooking. By C. K. TINKLER and M. C. SOAR 114

XVI. On the Presence of Amylase in Milk and Cheese. By M. SATO 120

XVII. The Antiscorbutic Requirements of the Monkey. By A. HARDEN and S. S. ZILVA 131

390072

PAGE

XVIII. The Production in Monkeys of Symptoms closely resembling those of Pellagra, by Prolonged Feeding on a Diet of Low Protein Content. By H. CHICK and E. M. HUME. (With Plate V and one figure) . . 135

XIX. On the Swelling of Gelatin in Hydrochloric Acid and Caustic Soda. By D. J. LLOYD. (With seven figures) 147

XX. The Anti-Scorbutic Properties of Concentrated Fruit Juices. Part III. By A. HARDEN and R. ROBISON 171

XXI. Observations on Anthocyanins. I: The Anthocyanins of the Young Leaves of the Grape Vine. By O. ROSENHEIM. (With one figure) 178

XXII. The Action of Thrombin upon Fibrinogen. By J. O. W. BARRATT. (With seven figures) 189

XXIII. Effect of Heat on the Anti-Scorbutic Accessory Factor of Vegetable and Fruit Juices. By E. M. DELF. (With four figures) . . 211

XXIV. The Products of the "Acetone: *n*-Butyl Alcohol" Fermentation of Carbohydrate Material with Special Reference to some of the Intermediate Substances produced. By J. REILLY, W. J. HICKINBOTTOM, F. R. HENLEY, and A. C. THAYSEN. (With two figures) . . . 229

XXV. A Note on Braunstein's Modification of the Mörner-Sjöqvist Process for the Estimation of Urea. By A. H. TODD 252

XXVI. The Iodometric Estimation of Sugars. By H. M. JUDD . 255

XXVII. Dietetic Experiments with Frogs. By A. HARDEN and S. S. ZILVA 263

Nos. 3 & 4 (JULY)

XXVIII. A Gas Analysis Apparatus Accurate to 0·001 % mainly designed for Respiratory Exchange Work. By A. KROGH. (With four figures) 267

XXIX. The Calibration, Accuracy and Use of Gas Meters. By A. KROGH. (With two figures) 282

XXX. The Relative Value of Fat and Carbohydrate as Sources of Muscular Energy. With Appendices on the Correlation between Standard Metabolism and the Respiratory Quotient during Rest and Work. By A. KROGH and J. LINDHARD. (With twenty-three figures) . . . 290

XXXI. Studies in the Acetone Concentration in Blood, Urine, and Alveolar Air. II: The Passage of Acetone and Aceto-Acetic Acid into the Urine. By E. M. P. WIDMARK. (With four figures) . . . 364

XXXII. Studies in the Acetone Concentration in Blood, Urine, and Alveolar Air. III: The Elimination of Acetone through the Lungs. By E. M. P. WIDMARK. (With three figures) 379

XXXIII. The Metabolism of Carbohydrates. Part I. Stereochemical Changes undergone by Equilibrated Solutions of Reducing Sugars in the Alimentary Canal and in the Peritoneal Cavity. By J. A. HEWITT and J. PRYDE. (With five figures) 395

XXXIV. The Existence in the Bile of an Inhibitor for Hepatic Esterase, and its Nature. By G. M. WISHART 406

XXXV. A Series of Abnormal Liesegang Stratifications. By E. HATSCHEK. (With Plate VI) 418

XXXVI. The Acidity of Ropy Milk. By K. FREEAR and E. C. V. VENN. (With seven figures) 422

PAGE

XXXVII. The Occurrence and Nature of the Plant Growth-Promoting Substances in Various Organic Manurial Composts. By F. A. Mockeridge 432

XXXVIII. Rapid Volumetric Methods for the Estimation of Amino-Acids, Organic Acids and Organic Bases. By F. W. Foreman. (With one figure) 451

XXXIX. Adsorptive Stratification in Gels. IV. By S. C. Bradford. (With Plates VII, VIII and one figure) 474

XL. The Nature of Yeast Fat. By I. Smedley MacLean and E. M. Thomas 483

XLI. The Extraction of the Fat-Soluble Factor of Cabbage and Carrot by Solvents. By S. S. Zilva. (With seven figures) 494

XLII. A Contribution to the Study of Keratomalacia among Rats. By M. Stephenson and A. B. Clark. (With Plates IX, X and eight figures) 502

XLIII. Barger's Microscopical Method of determining Molecular Weights. Part II. Its Application to Caseinogen. By K. Yamakami . 522

No. 5 (October)

XLIV. Oxidising Enzymes. II: The Nature of the Enzymes associated with Certain Direct Oxidising Systems in Plants. By M. W. Onslow 535

XLV. Oxidising Enzymes. III: The Oxidising Enzymes of some Common Fruits. By M. W. Onslow 541

XLVI. A Study of some Biochemical Tests. No. 2: The Adamkiewicz Protein Reaction. The Mechanism of the Hopkins-Cole Test for Tryptophan. A New Colour Test for Glyoxylic Acid. By W. R. Fearon . . 548

XLVII. The Heat-Inactivation of Diphtheria Antitoxin. By A. Homer 565

XLVIII. Note on "Scurvy" in Pigs. By R. H. A. Plimmer . . 570

XLIX. Note on the Oxidation of Quinine with Hydrogen Peroxide. By M. Nierenstein 572

L. Glycine and its Neutral Salt Addition Compounds. By H. King and A. D. Palmer 574

LI. Note on the Production of a Contracting Clot in a Gel of Gelatin at the Isoelectric Point. By D. Jordan Lloyd. (With Plate XI) . . 584

LII. An Experimental Study of the Effect of certain Organic and Inorganic Substances on the Bread-making Properties of Flour and on the Fermentation of Yeast. By H. Masters and M. Maughan . . 586

LIII. Ammonia Excretion, Amino-acid Excretion and the Alkaline Tide in Singapore. By J. A. Campbell. (With three charts) . . 603

LIV. Cuorin. By H. MacLean and W. J. Griffiths 615

LV. Factors influencing Alkaloidal Content and Yield of Latex in the Opium Poppy (*Papaver somniferum*). By H. E. Annett . . . 618

LVI. Digestibility of Germinated Beans. By D. M. Adkins . . 637

LVII. The Effect of Pyruvates, Aldehydes and Methylene Blue on the Fermentation of Glucose by Yeast Juice and Zymin in Presence of Phosphate. By A. Harden and F. R. Henley. (With two figures) . 642

PAGE

LVIII. The Distribution of Inorganic Iron in Plant and Animal Tissues. By H. W. Jones 654

LIX. The Nomenclature of the so-called Accessory Food Factors (Vitamins). By J. C. Drummond 660

LX. Researches on the Fat-soluble Accessory Substance. III: Technique for carrying out Feeding Tests for Vitamin A (Fat-soluble A). By J. C. Drummond and K. H. Coward 661

LXI. Researches on the Fat-soluble Accessory Substance. IV: Nuts as a Source of Vitamin A. By K. H. Coward and J. C. Drummond . 665

LXII. Researches on the Fat-soluble Accessory Substance. V: The Nutritive Value of Animal and Vegetable Oils and Fats considered in Relation to their Colour. By J. C. Drummond and K. H. Coward. (With four figures) 668

No. 6 (December)

LXIII. A Modification of the Barcroft and Winterstein Microrespirometers. By N. K. Adam. (With one figure) 679

LXIV. The Fermentation of Cellulose in the Paunch of the Ox and its Significance in Metabolism Experiments. By A. Krogh and H. O. Schmit-Jensen 686

LXV. A Method for Obtaining Uncontaminated Specimens of Urine from the Billy Goat; with some Notes upon the Normal Metabolism of this Animal. By R. A. Peters. (With six figures) 697

LXVI. The Action of Sea Water on Cotton and other Textile Fibres. By C. Dorée 709

LXVII. A Note on the Differentiation of the Yellow Plant Pigments from the Fat-Soluble Vitamine. By M. Stephenson. (With one chart) . 715

LXVIII. Note on the Vitamine Content of Milk. By F. G. Hopkins. (With four figures) 721

LXIX. The Effects of Heat and Aeration upon the Fat-soluble Vitamine. By F. G. Hopkins. (With five figures) 725

LXX. Researches on the Fat-soluble Accessory Factor (Vitamin A). VI: Effect of Heat and Oxygen on the Nutritive Value of Butter. By J. C. Drummond and K. H. Coward. (With three figures) 734

LXXI. The Action of Ozone on the Fat-soluble Factor in Fats. Preliminary Note. By S. S. Zilva 740

LXXII. The Nutritive Value of Lard. By J. C. Drummond, J. Golding, S. S. Zilva and K. H. Coward. (With seven figures and Plate XII) 742

LXXIII. The Iodimetric Estimation of Sugars. By J. L. Baker and H. F. E. Hulton 754

LXXIV. The "Ammonia Coefficient" of Pregnancy. By W. C. Cullis and E. E. Hewer. (With three charts) 757

LXXV. The Penetration of Electrolytes into Gels. II: The Application of Fourier's Linear Diffusion Law. By G. S. Adair 762

Index 781

OBITUARY NOTICE.

ADRIAN JOHN BROWN.

BIOCHEMISTRY has suffered a severe loss by the sudden death of ADRIAN J. BROWN, which followed closely upon that of his wife last July. Adrian Brown was born in 1852 at Burton and educated at the local Grammar School, whilst he received his chemical training at the Royal School of Mines under Frankland. After acting for a short time as private assistant to Dr Russell at Bartholomew's Hospital he was appointed chemist to the brewing firm of Messrs Thos. Salt & Co. of Burton-on-Trent, where he remained until 1899. In that year he was appointed to the newly-founded Chair of Malting and Brewing in Birmingham, and in 1904 became Professor of the Biology and Chemistry of Fermentation and Director of the School of Brewing, posts which he occupied until his death. His time at Birmingham was largely occupied with his professional duties and he trained a large number of students, many of whom entered the brewing industry. He was a most genial and sympathetic teacher taking great pains with his students, by whom he was greatly respected and beloved. In connection with his teaching he published *Laboratory Studies for Brewing Students* (1904), a practical text-book of much value. He was the first Examiner in Biological Chemistry to the Institute of Chemistry, the recognition of this subject in 1901 as an avenue of approach to the Institute marking an important stage in the development of Biochemistry in this country. He was an early member of the Biochemical Club, served on the committee, presided over the meeting held in Birmingham in 1912 and always took the greatest interest in the proceedings of the Club and of the Society into which it naturally developed. He was elected a Fellow of the Royal Society in 1911 and was President of the Institute of Brewing from 1917–1919.

He had a delightful home at Northfield, outside Birmingham, and was never happier than when discussing the treasures of his garden with a guest or demonstrating to him the abnormal production of marsh gas in the pond.

As an enthusiastic teacher and investigator in the laboratory he commanded the respect and admiration of all who came into intimate contact with him whilst his geniality of character and charming personality endeared him to a large circle of friends.

Adrian Brown was one of the circle of scientific men engaged in the brewing industry at Burton-on-Trent in the seventies of last century, of which such a charming and interesting account has been given by his brother, Horace T. Brown, in his *Reminiscences*. His own chief contributions to the scientific production of the "Bacterium Club" consisted in the study of two oxidising

organisms, *B. aceti* and *B. xylinum*, and a series of researches on the conditions of reproduction and fermentation of yeast.

One of the most important results of his investigation of *B. aceti* and *B. xylinum* (obtained from "mother of vinegar") was the clear demonstration that their oxidising action was strictly selective. Both of these organisms were shown to oxidise dextrose to gluconic acid, whereas they were without action on laevulose. Mannitol, on the other hand, was converted into laevulose, which was not further affected. *B. xylinum* was distinguished by producing a very bulky membranous growth, which appeared to be composed of cellulose, and which was made alike from dextrose and laevulose, and yielded a dextrorotatory reducing substance on hydrolysis. Subsequent investigations by O. Emmerling (1899) showed that the membrane in addition to cellulose contained a nitrogenous substance which yielded glucosamine on hydrolysis.

The investigation on the reproduction and fermentation of yeast also led to highly important results. In the first place he discovered the remarkable fact that when a medium is inoculated with yeast, reproduction ceases when a certain definite maximum number of cells is present and that if a number in excess of this be introduced *ab initio*, no increase whatever occurs, the added cells showing no signs of budding. This phenomenon was subsequently (1905) traced to the curious relations which subsist between yeast and oxygen. A certain concentration of oxygen in the cell appears to be necessary for the occurrence of cell division, and if the number of cells added is so great that this concentration is not reached no growth occurs. When a smaller number of cells is employed, these appear to take up the dissolved oxygen from the medium and if the growth is then allowed to proceed in the ordinary way (*i.e.* practically anaerobically in presence of the carbon dioxide produced by fermentation of the sugar in the medium) a certain definite number of cells is ultimately produced, whatever the seeding has been, "for the amount of available oxygen at the commencement of the experiments is constant, and it is conceivable that its power of stimulation is constant and governs the power of reproduction of the cells as a whole, irrespective of the number present." If the culture is continuously aerated throughout growth, the number of cells produced is much larger. He further demonstrated that Pasteur's conclusion that fermentation was life without air was not correct with respect to yeast, for in presence of abundant oxygen more and not less sugar was fermented than in its absence by equal numbers of yeast cells. A masterly criticism of Pasteur's experiments conclusively showed that these did not lead to a true expression of the fermentative powers of the yeast employed and that there was accordingly no experimental evidence to show that the fermentation of sugar by yeast was not independent of the presence or absence of oxygen in the surrounding medium. Three years later the discovery of zymase by Buchner brilliantly confirmed this conclusion and afforded a simple explanation of the facts. It was no doubt the observation, made in the course of these experiments, that the rate of fermentation of

sugar by a given number of yeast cells was, over a large range (5–20 %), independent of the concentration of the sugar, that led Brown to study the action of invertase, for which the ordinary law of mass action had been found to apply by O'Sullivan and Tompson in 1890. Investigation showed him that in reality the invertase behaved like the yeast cells, the initial rate of fermentation being constant over a considerable range of concentration, but that the products of the inversion inhibited the action of the enzyme and caused the pseudo-monomolecular course of the change. It was to explain this constancy of initial rate that he introduced the idea that combination occurred between the enzyme and substrate before the latter was decomposed, and lasted for a definite interval of time.

Perhaps the most interesting of all his scientific work was that occasioned by his discovery in 1907 that the seed of the barley is provided with a remarkable semi-permeable membrane. He noticed that the seeds of "blue" barley (*Hordeum vulgare* var. *caerulescens*) when steeped in dilute sulphuric acid became soft and swollen but retained their blue colour, although the blue pigment, which is situated in the aleurone cells, is readily turned red by acid. To quote his own words, this showed "that the covering has the property of resisting the passage of the acid, whilst it allows water to diffuse freely into the interior of the grain. So much is this the case that a dilute solution of sulphuric acid may be concentrated by steeping barley in it." Normal sulphuric acid was in fact thus concentrated from 49 to 76 g. of H_2SO_4 per litre. The resistance of the membrane to so concentrated an acid is very remarkable and a study of its behaviour to other substances, showed that the property was selective, substances like alcohol and acetic acid passing freely through the membrane, whilst salts in general, cane-sugar and tartaric acid could not penetrate it. The rate and extent of absorption of water from solutions of various substances could readily be measured and in short, in the words of Armstrong, an exquisite and invaluable method of studying the osmotic effect had been devised. The subject was pursued further in conjunction with Worley and Tinker more particularly in its physical aspects.

In addition to the work briefly outlined in the foregoing Brown made a number of investigations on various subjects including the heat of fermentation, the inheritance of colour in barley grains, *Bacillus subtilis*, and in conjunction with Ward on the antiseptic properties of hops.

It is characteristic of the man that although his scientific output was not large in amount, it dealt with problems of a fundamental nature and led in every instance to some discovery of real importance and value.

ARTHUR HARDEN.

I. ESTIMATION OF CARBON DIOXIDE, OXYGEN AND COMBUSTIBLE GASES BY KROGH'S METHOD OF MICRO-ANALYSIS.

By HANS OLUF SCHMIT-JENSEN.

From the Laboratory of Zoophysiology, University of Copenhagen.

(*Received November 14th, 1919.*)

Introduction.

For the study of respiratory processes of small organisms and for many other biological purposes it is essential to have methods for analysing small air-bubbles. In 1907 two such methods were described by August Krogh [1908], viz. a method of microscopical gas-analysis, applicable to air-bubbles of 1·00–0·01 mm.^{3}, and a method of micro-analysis for volumes between 1 and 20 mm.^{3}.

The principle of the latter method is based upon the fact that small air-bubbles placed in a fluid behave much as semi-fluid fat drops and easily allow transport from one place to another without disunion. For analysis an air-bubble is sucked up into a capillary tube of uniform calibre about 0·25 mm. in diameter and measured. A length of 100 mm. corresponds to a volume of about 5 mm.^{3}. Gas-absorptions are next performed one after the other in a funnel at the lower end of the capillary tube. Each absorption process being finished the air-bubble is sucked into the capillary tube again and measured anew. From the difference in length before and after absorption the absorbed quantity may be calculated. As the technique has been described by Krogh [1908, 1915] elsewhere in all details, I shall refer to these publications for further particulars.

The method is applicable to estimations of carbon dioxide and especially oxygen. Krogh has also applied the apparatus for the absorption-analysis of carbon monoxide (unpublished experiments).

In the autumn of 1916 I applied to Professor Krogh in order to obtain analyses of some gas-samples originating from gas cysts of the intestines of pigs, a pathological question I was engaged in studying. It soon turned out however that satisfactory results could not be obtained by the ordinary macro-gas-analysis, each estimation requiring samples from numerous cysts. Consequently Professor Krogh advised me to try micro-analysis of the contents of each single little cyst. As estimations of hydrogen and eventually methane were essential for the solution of the question under consideration

methods for micro-analysis of these gases had to be prepared. The results of these efforts are given below.

My best thanks are due to Professor August Krogh for his steady interest and help but for which I should not have been able to overcome the many difficulties met with during the work.

I also wish to express my sincere thanks to cand. pharm. K. L. Gad-Andresen, assistant at the laboratory, for numerous control-analyses by a Haldane-apparatus of the different gas-mixtures used for the micro-analyses.

Absorption-Analysis of Hydrogen.

It is well known that palladium, especially in colloidal form, is a good absorbing agent for hydrogen. Paal and Hartmann [1910] recommend a mixture of colloidal palladium and sodium picrate in watery solution for gas volumetric estimations of hydrogen. In this mixture palladium will act as a catalyst and rapidly transmit the hydrogen to the picric acid which is changed into 2.4.6-triaminophenol according to the formula:

$$C_6H_2.(NO_2)_3.OH + 9H_2 = C_6H_2.(NH_2)_3.OH + 6H_2O.$$

1 g. picric acid consumes 834 cc. hydrogen for complete reduction.

There could be little doubt that this reagent would be well adapted to micro-analysis and the results obtained answered our expectations.

The preparation can under ordinary conditions be procured ready for use from Kalle & Co., Biebrich a. Rh. Paal and Hartmann state that Kalle's preparation even exceeds their own in absorbing power. I could not however at that time procure the reagent from Kalle and had consequently to prepare colloidal palladium myself according to the methods of Paal and Amberger [1904].

The preparation was ready for use in February 1917; it should theoretically contain about 45 % palladium, but as the palladium chloride used did not dissolve quantitatively the percentage was supposed to be about 35 % only.

The following solution of this preparation was now made according to the formula marked *A* in Paal and Hartmann's publication [1910, p. 249], the lower palladium percentage of the present preparation being considered:

0·66 g. colloidal palladium,
0·42 g. sodium picrate,
20·00 g. distilled water.

The solution was prepared on September 26th, 1917, and in October and November the same year was used for the analyses given below. The solution does not keep its activity for long periods; and half a year later, in May 1918, it absorbed only very slowly and anything but quantitatively. Colloidal palladium in substance also gradually loses its solubility.

Three series of analyses of different hydrogen mixtures were made by means of this palladium solution. The apparatus used had a capillary tube of uniform calibre about 0·25 mm. in diameter. The tube as well as the funnels

were filled with distilled water, slightly acidulated by means of sulphuric acid, a precaution observed once for all for the sake of carbon dioxide but of no importance for other gases. The samples to be analysed were prepared by mixing a certain quantity of hydrogen with an ample amount of atmospheric air in a 100 cc. gas-sampling-tube. Each mixture was analysed in a Haldane-apparatus to establish the exact composition. The gas was led from the sampling tube through a narrow leaden tube to the funnel, which was for safety's sake immersed in water until the air-bubble had been taken in. The bubbles analysed had a volume of 5 $mm.^3$ on an average. The length of the bubble being measured the funnel was filled with alkaline pyrogallate solution. Oxygen has to be removed before hydrogen absorption owing to the fact that palladium catalyses oxygen and hydrogen with formation of H_2O. After the estimation of oxygen the funnel was filled with palladium mixture and hydrogen absorbed and determined.

It soon turned out that it was highly essential to maintain definite durations of the two absorption processes in order to obtain uniform results, partly to avoid loss of hydrogen by diffusion during oxygen absorption, partly to secure a complete absorption of hydrogen. A stop watch was advantageously used for this purpose and is on the whole much to be recommended to secure a uniform course of micro-analyses, where diffusion plays a part unknown in ordinary gas-analysis.

It was found in analyses of atmospheric air that 1 *minute* will suffice for a complete absorption of oxygen provided the bubble is moved up and down in the lower parts of the capillary tube twice or thrice during the process.

With regard to hydrogen absorption by the palladium mixture used it was established that $2\frac{1}{2}$ *minutes* are enough to secure a complete absorption, provided the bubble is moved up and down in the lower parts of the tube *incessantly* during absorption.

Before giving the results I shall briefly mention a source of error which at the beginning caused many fallacious results. Accidentally the cause of these errors was detected when on one occasion I happened to keep the funnel under observation through the lens at the moment when pyrogallate solution was added. In the funnel was left as small a quantity of water as possible in order to exclude supersaturation when water and pyrogallate solution were mixed and the lower end of the air-bubble was consequently found very near the point where the bore widens into the funnel. At the moment when water and pyrogallate solution ran into one another I saw the end of the bubble break and the meniscus draw a little backwards into the capillary tube. This observation taken together with the result of the calculation—the oxygen percentage far too high, the hydrogen percentage far too low—cleared up the matter, a minimal part of the sample had been lost in the funnel. The phenomenon was now studied a little further and it was found that bubbles of atmospheric air never broke under conditions such that mixtures of hydrogen broke almost constantly. Furthermore it was established that air-bubbles will

break the easier the higher their percentage of hydrogen. The phenomenon is not seen when palladium mixture is added.

It is therefore essential always to keep the lower end of an air-bubble one to two millimetres within the mouth of the capillary tube when pyrogallate solution is added. As soon as this precaution was observed the phenomenon disappeared totally.

Series no. 1. 18. x. 17.

Percentage of

O_2: 11·2, 11·4, 11·0, 11·0, 10·9, 11·3, 11·5, 11·2, 11·4, 11·2. Mean 11·21.

σ*: $\pm$ 0·2, σ_m†: $\pm$ 0·06. Systematic error: $-$ 0·06 $\pm$ 0·06. Macro-analysis 18. x.: 11·15 % O_2; 19. x.: 11·15 % O_2.

H_2: 46·9, 46·8, 46·9, 46·9, 47·2, 46·7, 46·8, 46·9, 46·6, 46·9. Mean 46·86.

σ: $\pm$ 0·16, σ_m: $\pm$0·05. Systematic error: +0·81 $\pm$ 0·05. Macro-analysis 18. x.: 47·67 % H_2; 19. x.: 47·67 % H_2.

* σ = standard deviation. † σ_m = standard deviation of mean.

Series no. 2. 1. xi. 17.

Percentage of

O_2: 13·7, 13·6, 13·5, 13·8, 13·9, 13·6. Mean: 13·68.

σ: $\pm$ 0·15, σ_m: $\pm$ 0·06. Systematic error: —. Macro-analysis 1. xi. 17: —.

H_2: 35·7, 35·9, 35·9, 35·5, 35·6, 35·6. Mean: 35·70.

σ: $\pm$ 0·17, σ_m: $\pm$ 0·07. Systematic error: + 0·49 $\pm$ 0·07. Macro-analysis 1. xi. 17: 36·19 % H_2.

Series no. 3. 2. xi. 17.

Percentage of

O_2: 17·8, 17·8, 17·7, 17·8, 17·5, 17·5, 17·9, 17·8, 17·6, 18·0. Mean: 17·74.

σ: $\pm$ 0·13, σ_m: $\pm$ 0·04. Systematic error: +0·13 $\pm$ 0·04. Macro-analysis 3. xi.: 17·87 % O_2.

H_2: 15·3, 15·3, 15·3, 15·0, 15·6, 15·4, 15·3, 15·3, 15·2, 14·9. Mean: 15·26.

σ: $\pm$ 0·2, σ_m: $\pm$ 0·06. Systematic error: +0·13 $\pm$ 0·06. Macro-analysis 3. xi.: 15·39 % H_2.

A modified Micro-Analysis Apparatus with Combustion Funnel.

The only technical difference between this apparatus and the ordinary one as used for the hydrogen analyses is, beside the combustion funnel, the increase of the diameter of the capillary tube to about 1 mm. In the apparatus employed the capillary tube was found to be of uniform calibre, a length of 100 mm. corresponding to a volume of about 85 mm.^{3}, about 17 times as much as the same length represents in a 0·25 mm. capillary tube.

The objects of this essential innovation were these:

1. To obtain air-bubbles so big that it might be possible to insert a platinum wire into them for combustion analysis. It had proved easy enough to construct platinum instruments small enough for air-bubbles of a volume of only 5 mm.^{3} but the insertion of them into such a small bubble was made impossible by the surface tension, the bubble escaping when meeting the wire.

2. To obtain better carbon dioxide analyses, the results by means of the ordinary apparatus being far too irregular, affected as they were by considerable accidental as well as systematic errors.

I soon became aware of several peculiarities which had to be considered when accurate results were to be obtained by this apparatus.

1. The air-bubble has to be sucked into the capillary tube for measuring purposes with *uniform speed* during a given analysis. The reason for this precaution is that the thickness of the film of water adhering to the inside of the capillary tube depends greatly upon the speed at which the air-bubble is moved upwards. If the bubble is moved too quickly the upper meniscus will not get time enough for pushing the fluid in front of it, the film will be relatively thick and the bubble in question will increase in length as compared with a measurement after a slower movement. This phenomenon is of no importance in a 0·25 mm. tube.

2. A deduction has to be made, as the volumes of gas-bubbles are not strictly proportional to their measured lengths owing to the spherical ends of gas-bubbles in capillary tubes. The deduction from each measured length was calculated to be 0·3 mm. when the diameter of the tube is about 1 mm. The corresponding deduction in a tube of 0·25 mm. is but 0·08 mm., a correction so small that it can be omitted in all ordinary circumstances. For further particulars *vide* Krogh [1908].

Example: estimation of oxygen in atmospheric air:

$$\left.\begin{array}{l} 13{\cdot}3^\circ\ \text{C. } 71{\cdot}9\ \text{mm., corrected length } 71{\cdot}6 \\ 13{\cdot}4^\circ\ \text{C. } 56{\cdot}9\ \text{,,} \qquad \text{,,} \qquad 56{\cdot}6 \end{array}\right\} 15{\cdot}0 = 21{\cdot}0\ \%.$$

3. The absorption conditions differ from those in the small apparatus, where a bubble of the size of a millet grain is almost completely surrounded by the absorbing reagent. A bubble of 100–150 mm.^{3} on the contrary has the size of a small pea and will consequently occupy about half the capacity of the absorbing funnel of this apparatus, for which reason absorption has to take place from the lower meniscus and the thin films of fluid adhering to the inside of the part of the funnel filled with gas. The absorption conditions do not seem to be less favourable than was the case in the smaller apparatus, but an essential requirement for quick and complete absorptions is that the gas sample is sucked back once or twice into the capillary tube so that the fluid adhering to the inside of the upper part of the funnel may be renewed. The considerable volume of the sample renders it necessary that an equal volume of the absorbing agent must drop out of the funnel; it has to be replaced subsequently when the gas is sucked up into the capillary tube again (compensation by the same reagent as water might cause supersaturation). Three-quarters of a minute secures a complete absorption of oxygen in an analysis of atmospheric air.

When these precautions were first taken the apparatus worked as well as the ordinary one. A detail which may save some time is yet to be mentioned. Instead of measuring the length from the ordinary zero lying about 3·5 cm. from the funnel I have chosen a division about 0·5 cm. from the funnel (Fig. 1, *A*, *c*) with the advantage that the samples to be analysed may be 20 mm. longer and that water as well as potash may be sucked up to this point without contaminating much of the capillary tube.

In the calculation of the results the temperature-correction is introduced simply by deducting or adding 0·1 % of a measured length for every 0·2° the temperature has changed.

A *combustion funnel* for this apparatus was constructed to render micro-analysis of combustible gases possible. For technical particulars I refer to the following chapter where the final shape of the entire arrangement for micro-analysis will be described.

Carbon Monoxide.

Two series of estimations were made, one by combustion and one by absorption. The equation for combustion of this gas is:

$$2CO + O_2 = 2CO_2.$$

As the carbon dioxide formed cannot be measured with sufficient accuracy owing to diffusion, combustion has to be followed by absorption of CO_2 by potash and the CO calculated as two-thirds of the final contraction. It is consequently unnecessary to read off before the CO_2 has been absorbed. For absorption analyses acid cuprous chloride solution prepared according to the directions given by Winkler [1910] was used. As the process of absorption takes place rather slowly it is advisable to replace the reagent once during the absorption. Oxygen is also absorbed by this reagent and has therefore to be removed first by pyrogallate solution.

The gas-mixture employed was prepared by adding pure carbon monoxide to atmospheric air. The same mixture was used for both series.

Series no. 4 a. 3. v. 18.

Combustion analyses.

Capillary tube and both funnels filled with slightly acidulated distilled water. Volume of samples about 110 mm.^{3}. Five seconds' combustion at a heat just enough to make the platinum wire visibly glowing. Absorption of CO_2 by potash. CO = two-thirds contraction + CO_2.

Percentage of
CO: 20·6, 20·7, 20·7, 20·7, 20·5, 20·6, 20·5, 20·6, 20·7, 20·6. Mean: 20·62.
σ: ± 0·08, σ_m: ± 0·03.

Series no. 4 b. 3. v. 18.

Absorption analyses.

Apparatus filled with water as in series no. 4 *a*. Volume of samples about 71 mm.^{3}. Oxygen absorption by pyrogallate ¾ minute, the sample being moved up and down thrice. Carbon monoxide absorption: first portion of cuprous chloride solution = 1 minute; second portion of cuprous chloride solution = 2½–3 minutes; the sample being kept incessantly in motion the whole time.

Percentage of
O_2: 16·7, 16·8, 16·4, 16·6, 16·6, 16·7. Mean: 16·63.
σ: ± 0·14, σ_m: ± 0·06.
CO: 20·4, 20·4, 20·7, 20·5, 20·5, 20·4. Mean: 20·48.
σ: ± 0·12, σ_m: ± 0·05.

Macro-analysis of the gas-mixture was not made, as the Haldane-apparatus was temporarily out of order.

As will be seen the CO values of the absorption estimations are slightly lower than those obtained by combustion. The latter express no doubt the exact percentage of CO, a systematic error of any importance being improbable.

Hydrogen.

One series of combustion analyses was made. The equation is:

$$2H_2 + O_2 = 2H_2O,$$

(2 vol. + 1 vol = 0 vol.).

Hydrogen is calculated as two-thirds of the contraction.

Series no. 5. 6. v. 18.

The gas-mixture was prepared by adding pure hydrogen to atmospheric air. Capillary tube and funnels filled with acidulated water. 10 seconds' combustion as in series no. 4 *a*. Two-thirds × contraction = H_2.

Percentage of

H_2: 18·4, 18·4, 18·5, 18·6, 18·5, 18·5. Mean: 18·48.

σ: ± 0·09, σ_m: ± 0·04. Systematic error: +0·29 ± 0·04. Macro-analysis 6. v. 18: 18·77 % H_2.

Compare further hydrogen estimations in the following chapter.

Carbon Dioxide.

The difficulty of the micro-estimation of CO_2 is due to the enormous rapidity with which this gas diffuses in most fluids, and measures which impede loss by diffusion are necessary to secure satisfactory analytical results. To this end Krogh [1908] recommends either to fill the funnel with conc. acidulated glycerol or to collect the sample in the funnel of the analysis apparatus over mercury.

I have myself spent much time seeking a satisfactory solution of this problem. As my experiences may perhaps be of some use to others working on this line and as they in my opinion clearly illustrate the essential factors to be considered in the micro-estimation of CO_2 I shall sketch the different methods tried before good results were gained.

Loss by diffusion in the absorption funnel was first considered and this part of the apparatus filled either by mercury or by conc. acidulated glycerol. Mercury gave considerably higher percentages than those obtained over water, and somewhat better results than conc. glycerol but there was still a loss in the rest of the apparatus. To prevent this the upper parts of the apparatus were filled with a saturated solution of sodium chloride, acidulated by hydrochloric acid, the absorption funnel remaining filled with mercury. This salt solution was soon replaced by *a mixture of equal parts of glycerol and saturated sodium chloride solution* both acidulated by hydrochloric acid. *This mixture proved to be an important step forward and has ever since been used permanently in the apparatus for all gases.* In the sequel it will be named "*g.s. mixture.*"

The mixture in question is thin enough for the movements in a capillary tube 1 mm. in diameter, but requires to a still higher degree than water movements of uniform speed. Augmentation of the glycerol content cannot be recommended, a mixture of 75 %, being too thick, caused accidental errors up to 1 %.

It was now justifiable to omit the filling of the funnel with mercury as being both troublesome and time-wasting. As the whole apparatus for several reasons cannot be filled throughout with mercury a systematic error bigger or smaller will always be present in CO_2 analyses.

This error being constant it will hardly pay to spend much time on diminishing it below a reasonable size which can be attained without particular arrangements.

Three series of CO_2 estimations of different mixtures were now made by this improved technique. In these and all the following series the apparatus was entirely—capillary tube as well as absorption funnel—filled with "g.s. mixt."

Series no.	6	7	8
	(18. v. 18)	(21. v. 18)	(21. v. 18)
Percentage of	CO_2	CO_2	CO_2
	23·8	51·3	60·8
	23·8	51·8	60·7
	23·8	51·4	60·4
	23·8	51·6	60·6
	23·7	51·5	60·5
	23·8	51·5	60·8
	23·9	51·4	60·4
	23·9	51·3	60·9
	23·8	51·4	60·6
	24·1	51·4	60·5
Mean	23·84	51·46	60·62
σ	± 0·1	± 0·15	± 0·18
σ_m	± 0·03	± 0·05	± 0·06
Systematic error	+ 0·59 ± 0·03	+ 0·95 ± 0·05	+ 1·21 ± 0·06
Macro-analysis	24·43	52·41	61·83 % CO_2
	(18. v. 18)	(23. v. 18)	(18. v. 18)

The accidental errors in these series are evidently of no importance as compared with the systematic ones. The latter are not proportional as might be expected to the concentrations of CO_2, the lower percentages having a relatively higher systematic error than the higher percentages.

Compare on this point the CO_2 analyses of the next chapter.

The modified Micro-Analysis Apparatus combined with Clock-Work and Combustion Funnel.

This construction represents the final shape of the apparatus I have come to during my work. The arrangement will be readily understood from Fig. 1 and annexed explanations.

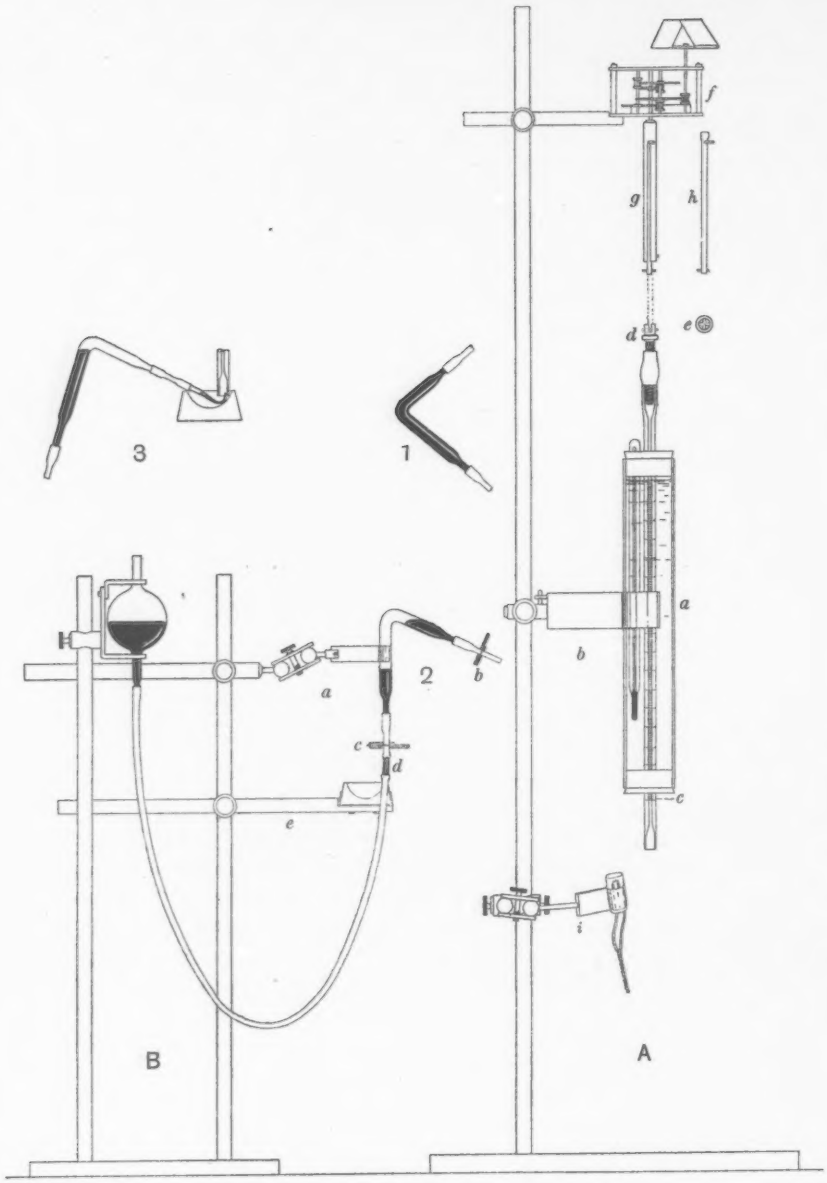

Fig. 1. About ¼ nat. size.

Analysis apparatus. The apparatus is, as to the capillary tube, identical with the one described in the preceding chapter, the diameter being about 1 mm. The clock-work has necessitated a little change. The side-tube has been removed and the screw inserted in the enlarged top funnel. The screw is tightened by a very stiff mixture of pure india-rubber and vaseline melted together. The tube is filled with "g.s. mixture." This fluid undergoing no bacterial decomposition the capillary tube keeps clean for months.

When the apparatus has to be used, the screw is wound as far down as possible, the "g.s. mixture" removed from the absorption funnel by means of a pipette and replaced by a fresh solution, a small quantity of which is sucked up into the capillary tube. By following these directions one may always be sure that the gas is brought into contact with a fluid of constant composition.

Owing to the wide diameter the tube is easily cleaned without the aid of an aspirator.

Clock-work. The object of the clock-work is to secure absolutely uniform speed when the gas is sucked up into the capillary tube for measurement. All movements towards the absorption funnel have to be performed by revolving

DESCRIPTION OF FIG. 1.

A. Entire arrangement for Micro-analysis.

a, capillary tube with water jacket and thermometer;

b, clamp and bosshead with arrangement for stopping in vertical position;

c, division taken as zero in measurements;

d, screw of vulcanite with brass top;

e, screw seen from the top with cruciform incision of the brass top;

f, clock-work with wings for regulating the speed;

g, metal tube fixed to the axle of the clock-work, containing a movable iron-pin to fit incisions of *d*;

h, iron-pin alone;

i, combustion funnel, carried by a clamp (compare *B*, *a*) adjustable to any angle. The conduction wires connect the platinum wire with accumulators and rheostat.

B. Stand for Micro-receivers.

a, elastic clamp with double ball-and-socket joint for fixation of micro-receiver;

b and *c*, clips on india-rubber tubing of micro-receiver;

d, piece of glass tube for disconnection from mercury reservoir;

e, clamp holding square glass pot with excavation for "g.s. mixture" for immersion of the absorption funnel of *A*, *a*.

1, position 1 of receiver, being used when the receiver has to be filled with mercury or when the last gas has to be driven out of the shorter limb for analysis;

2, position 2: gas has just been sucked in, receiver clipped at both ends and levelling-tube raised again;

3, position 3: gas from the longer limb is driven out through a narrow curved glass cannula into the absorption funnel, immersed in "g.s. mixture" (*e*). A drop of mercury regularly left in the drawn out part of the shorter limb is pushed in front of the gas so removing any foreign gas present.

the screw by hand. The most suitable speed for sucking in gas is about 100 mm. in 30 seconds. Twice the speed may very well be applied but the screw is, according to my experience, rather liable to become leaky after some time when revolved so quickly. The speed is regulated by means of a pair of wings adjustable to different angles. In order to secure the most uniform speed obtainable I have furthermore wound the clock-work before each measurement.

Coupling to the screw is effected in a few seconds and disconnecting as quickly. When the iron-pin is fixed in the top of its tube the clock-work never causes any trouble during the other manipulations of the analysis apparatus.

Combustion funnel (Fig. 1, *A*, *i*). This small instrument fixed by a clamp and a bosshead may be raised to the absorption funnel. The construction needs some explanation.

Two platinum wires, 4 cm. in length, 0·3 mm. in diameter, are flattened by a hammer at one end to an extent of about 1 mm. Incisions 1 mm. deep following the longitudinal axes of the wires are made in the middle of the flattened parts by means of a pair of scissors. The wires are insulated by platinum imbedding glass drawn out to thin threads and wound round the wires over the blowpipe. (Enamel might be tried for this purpose.) The insulation has to begin just beneath the flattened parts and must cover the wires to a length of about 3 cm. A cork, 8 mm. long, 15 mm. in diameter, is soaked in paraffin (melting point 60–70° C.) till all air is driven out of the pores. Two holes of the diameters of the insulated wires are bored near the centre of the cork 2 mm. apart from one another and the wires are inserted with the uninsulated ends first (no paraffin must touch the upper end). The flattened points have to be 15 mm. above the cork. A platinum wire 0·05 mm. in diameter is next fixed between the flattened points of the insulated wires acting as carriers of the current. Fixation is obtained by winding the wire many turns round the points. The length of the glowing-wire ought to be 5–6 mm., it may be shaped in the form of a single spiral winding for instance. Sharp curves must be avoided the wire being sure to fuse in such places. The advantage of making the wire amply long is that a broken wire may easily be welded together afterwards by simply crossing the ends till contact is obtained and increasing the current to a bright white heat. The whole arrangement allows of the replacement of a fused wire in a few minutes. The uninsulated ends of the wires on the other side of the cork are soldered to ordinary conducting wires connected to a four volt accumulator battery, a rheostat and a key.

The cork is now inserted into a piece of glass tube, 2·5 cm. long and 15 mm. in diameter. The funnel thus being formed is next filled with water (air-bubbles being avoided!) up to the glowing-wire and is now ready for use.

The rheostat may of course be of any construction at disposal, but it is essential to the task in question, that it can regulate from the slight heat of a not glowing-wire through just visible glowing to a bright white heat. A rheostat of 10 ohms fulfilled these requirements.

The heat of the glowing-wire must be regulated according to the different gases. For hydrogen the heat of a "dark" wire is sufficient, the extra heat produced at the combustion making the wire glow for a moment. For carbon monoxide a just visibly glowing-wire will do, while methane requires at least a bright red heat, the extra heat produced by the process causing the wire to glow momentarily at a bright white heat. I have often raised the temperature to a bright white heat during my methane analyses, but it is hardly essential and rather risky for the wire.

As the combustion processes under these conditions take place instantaneously it is neither necessary nor advisable to keep the wire aglow for more than five seconds. When the wire is inserted into an air-bubble and the temperature of the wire raised to a bright white heat during five seconds, the volume of the bubble will not suffer any change at all unless combustible gases are present. When the same procedure is extended to last ten seconds or more the bubble will increase slightly in volume owing to emigration of gas from the heated water in the funnel.

In estimating both hydrogen and methane the measurement after combustion of hydrogen may very well be done without removing the combustion funnel.

The course of a complete Analysis (CO_2, O_2, H_2, CH_4, N_2).

1. *Preparations.* The absorption funnel and capillary tube are filled with fresh "g.s. mixture" by means of a pipette and the screw as described before. The lower end of the funnel is inserted into the glycerol basin (Fig. 1, *B*, *e*). The clock-work is wound and connected to the screw (Fig. 1, *A*, *d*). The gas-sample is taken into the funnel and the clock-work started. When a sufficient amount of gas is sucked into the capillary tube, the clock-work is stopped, disconnected from the screw, the glycerol basin removed and the superfluous gas allowed to escape from the funnel by turning the apparatus. The lower meniscus of the sample being placed at zero by means of the restarted clock-work the first measurement is performed and noted together with the temperature of the water-jacket.

2. *Absorption of carbon dioxide.* The first measurement being finished the lower meniscus of the sample is taken down near the mouth of the funnel, "g.s. mixture" removed by a pipette and without any washing of the funnel replaced by 10 % potash. The sample is now taken down into the funnel, the upper meniscus being kept in the capillary tube but 2–3 mm. above the funnel. *It is an essential point that the fluid in the funnel must never come into contact with the "g.s. mixture" above the upper meniscus.* Absorption takes place instantaneously. As soon as the clock-work has been rewound and connected the sample is sucked back into the capillary tube together with a short column of potash below the lower meniscus stopped at zero. Second measurement. Temperature noted.

3. *Absorption of oxygen.* The second measurement being done the sample is taken down near to the funnel and the potash without any washing of the funnel

replaced by pyrogallate solution. Absorption-time about three-quarters of a minute (established beforehand for a given apparatus and pyrogallate solution by means of an oxygen estimation in atmospheric air!). The sample has to be moved up and down thrice to secure a complete absorption. The clock-work is wound anew and connected. The sucking in has to be stopped when the lower meniscus has reached a few millimetres within the capillary tube and any trace of pyrogallate is removed thoroughly from the funnel as well as the capillary tube by means of a pipette filled with distilled water. This being done the lower meniscus of the sample is taken down near the funnel and the water replaced by "g.s. mixt." The sample is next taken up to zero by means of the restarted clock-work. Third measurement. Temperature noted.

4. *Combustion of hydrogen.* The third measurement being done addition of a suitable excess of oxygen for the combustions has to take place. Either pure oxygen or atmospheric air can be applied; in the latter case it will often be necessary to remove part of the sample in order to make room for the necessary excess of oxygen (*vide* calculation below). Application of pure oxygen is advantageous as it generally allows the whole of the sample to be retained. The addition is performed in the following way. The lower meniscus of the sample is placed 1–2 mm. above the funnel, a bubble of oxygen taken into the funnel and sucked up—the screw being turned by hand—into the capillary tube below the sample but *separated* from it by a short column of "g.s. mixture." When a sufficient amount is sucked in, the apparatus is turned so that superfluous oxygen may escape from the funnel. The two air-columns are now quickly taken down into the funnel and united there. The rewound clock-work is started and the enlarged sample taken up. Fourth measurement. The difference between the fourth and third measurements represents consequently the oxygen added. The lower meniscus is next placed near the funnel and a thorough washing of the funnel—inside as well as outside—performed in order to remove any trace of glycerol. The lower 4–5 mm. of the capillary tube are equally washed by moving the screw up and down. The air-bubble may now be taken down into the absorption funnel the upper meniscus being kept 2–3 mm. within the capillary tube as emphasised before, the combustion funnel filled with water is cautiously raised, so that the glowing-wire is found in the centre of the bubble, and a current sent through the wire *without* making it glow visibly. If hydrogen be present the wire will glow up for a fraction of a second. The current is broken off by the key as soon as this has taken place and the sample is sucked up by clock-work without removing the combustion funnel. Fifth measurement. Temperature noted. Two-thirds of the contraction is hydrogen.

5. *Combustion of methane.* The fifth measurement being made the sample is taken down into the funnel again, the glowing-wire eventually centred anew in the bubble and the current *gradually* increased until the wire reaches a bright red heat. The heat should be increased cautiously as otherwise the wire may fuse owing to the extra heat produced at first by the combustion.

If methane be present the wire will for an instant glow up from a bright red to a bright white heat. The combustion is now finished, the combustion funnel removed and a small amount of potash cautiously added by means of a curved pipette to the water in the funnel so as to absorb the carbon dioxide produced by the combustion process. The clock-work is wound and started. Sixth and final measurement. Temperature noted. One-third of the contraction is methane.

6. *Nitrogen.* Calculated as residue.

Calculation. The calculation of the percentages, easily performed by a slide rule, does not require to be specially mentioned except in a case where the sample has been diminished during an analysis in order to make room for atmospheric air for combustion.

The new volume of the sample must consequently be corrected for the carbon dioxide and oxygen removed at the beginning of the analysis. The following example will explain the case.

Reading No.	Temp. ° C.	Length	Correct. length	Diff.	%
1.	13·6	110·6	110·3		
2. CO_2 absorbed	13·8	102·4	102·0	8·3	7·5 CO_2
3. O_2 absorbed	13·8	101·7	101·3	0·7	0·65 O_2
4. *Sample diminished to*	13·9	32·2	31·9		
5. Plus atmosph. air	13·9	147·3	147·0	2·1	4·0 H_2
6. Combustion of H_2	14·0	145·2	144·9	21·6	20·7 CH_4
7. Combustion of CH_4 plus CO_2 absorbed	14·1	123·7	123·3		

$$CO_2 = \frac{100 \times 8{\cdot}3}{110{\cdot}3} = 7{\cdot}5\ \%,$$

$$O_2 = \frac{100 \times 0{\cdot}7}{110{\cdot}3} = 0{\cdot}63\ \%.$$

Correction of reading no. 4:

$$31{\cdot}9 \times \frac{110{\cdot}3}{101{\cdot}3} = 34{\cdot}7 \text{ mm.}$$

$$H_2 = \frac{100 \times 2{\cdot}1 \times 2}{34{\cdot}7 \times 3} = 4{\cdot}0\ \%,$$

$$CH_4 = \frac{100 \times 21{\cdot}6 \times 1}{34{\cdot}7 \times 3} = 20{\cdot}7\ \%.$$

Carbon Dioxide.

The gas-mixtures for these series were prepared of pure CO_2 and atmospheric air mixed in a 100 cc. receiver. As the clamp (Fig. 1, *B*, *e*) for the glycerol basin was not ready when these analyses were made the clamp carrying the analysis apparatus had to be taken out of its bosshead down to a basin with glycerol on the table. The absorption funnel being quite filled with gas, the apparatus and the basin were raised together. The basin was not removed till the sample had been sucked up into the capillary tube. In order to avoid this troublesome and time-wasting removal of the analysis apparatus from the stand the clamp (Fig. 1, *B*, *e*) was constructed. The glycerol basin is thereby raised to the absorption funnel and fixed before the sample is taken into the funnel.

Series no.	9 (28. v. 19)	10 (30. v. 19)	11 (18. vi. 19)
Percentage of	CO_2	CO_2	CO_2
	—	21·8	—
	11·3	21·8	—
	11·0	22·0	—
	11·0	21·9	32·8
	11·4	22·0	32·9
	11·0	21·8	33·0
	11·0	21·8	33·0
	11·0	22·0	33·0
	11·2	21·9	33·0
	10·9	22·0	33·0
	11·3	21·9	32·8
	11·1	21·9	33·0
	11·2	21·9	33·0
Mean	11·12	21·90	32·95
σ	± 0·16	± 0·08	± 0·09
σ_m	± 0·05	± 0·02	± 0·03
Systematic error	+ 0·58 ± 0·05	+ 0·71 ± 0·02	?
Macro-analysis	11·70 % (28. v. 19)	22·61 % (30. v. 19)	(lost)

The *accidental errors* of these analyses have as it may be seen been reduced to such a minimum, that they may wholly be attributed to the unavoidable errors of reading. When the length of an air-bubble is about 100 mm. an error of ± 0·1 mm. corresponds to ± 0·1 %.

The *systematic errors* ought theoretically to be strictly proportional to the percentages of CO_2, found in these analyses. This proportionality is neither absolute in these series nor in the series no. 6, 7 and 8 of the preceding chapter, the tendency being that the lower percentages have a relatively higher systematic error than the higher ones. I have reason to believe that this condition will be still more pronounced at CO_2 percentages higher than the 22·6 % of series no. 10, but as such percentages are not commonly met with during biological work I have not tested the point. The training of the analyst is no doubt of great importance when dealing with a gas diffusing so rapidly and it is to be recommended that every operator should establish his individual systematic error as regards different CO_2 percentages. This is done by means of a series of micro-analyses and a macro-analysis of each CO_2-mixture. The use of the fixable glycerol-pot, mentioned above, might possibly have given more uniform errors in the present series.

The fact that the errors of these two series are considerably higher than that of the series no. 6 is no doubt wholly due to the slower movement of the clock-work, more time being left for diffusion in the funnel.

Hydrogen.

The gas-mixture used for the analyses given below was prepared by adding pure hydrogen to atmospheric air. It proved exceedingly explosive, many analyses being spoiled at the beginning. Good results were obtained, when the

current was interrupted instantaneously when the slightest trace of contraction was seen. The wire would nevertheless glow up and the combustion proceed quietly.

The oxygen percentages given are calculated by adding a third of the contraction to the values found by pyrogallate absorptions after the combustions.

A series of absorption analyses was also made, the absorbing agent being the palladium mixture mentioned at the beginning of this paper. The results proved contrary to my expectations very bad. The loss of hydrogen by diffusion during oxygen absorption was both considerable and inconstant. This method was therefore given up as inapplicable, the combustion method being far superior to it.

Series no. 12. 18. iii. 19.

Percentage of

H_2: 21·0, 21·0, 20·9, 20·8, 20·9, 20·7. Mean: 20·88.

σ: ± 0·12, σ_m: ± 0·05. Systematic error: +0·12 ± 0·05. Macro-analysis (18. iii.): 21·0 % H_2.

O_2 (see text above): 16·6, 16·6, 16·8, 16·7, 16·8, 16·7. Mean: 16·70.

σ: ± 0·09, σ_m: ± 0·04. Systematic error: (−0·2 ± 0·04). Macro-analysis (18. iii.): (16·5 % O_2 by calculation).

The accidental and systematic errors are equal.

In all ordinary circumstances, where the utmost accuracy is not wanted, one may take for granted that there is no systematic error upon analyses of hydrogen, when the percentage does not exceed about 20.

Methane.

As it proved difficult to prepare a methane pure enough for gas-analysis by chemical methods, the gas was procured from nature in the following way.

A big glass flask was nearly filled by a mixture of water and the fresh contents from the paunch of a cow. The flask was alternately saturated by CO_2 and evacuated. When the saturation was supposed to be as complete as attainable the flask was placed at 37° C. for 24 hours. A violent fermentation began after some hours, the gas produced being collected in a large receiver. The gas was freed from CO_2 by means of a Pettenkofer's absorption tube with potash. Macro-analysis of the gas so procured showed 92·9 % methane and 7·1 % nitrogen, the CO_2 formed in the combustion process being exactly equal to half of the contraction.

This concentrated methane mixture was used for the series to be given below. The plan of this series is somewhat different from that hitherto followed. It was namely thought desirable to imitate the natural conditions as closely as possible, where oxygen or atmospheric air generally has to be added to the sample. To this end small amounts of the methane mixture were measured in the analysis apparatus, whereupon atmospheric air or pure oxygen was added. Explosions were not seen in any case.

The oxygen was generated by means of a wash bottle containing oxylith on to which water was dropped. The addition of pure oxygen is most advantageous, as it allows of taking in much bigger samples than are possible when

atmospheric air is used. An oxygen-generator ought therefore always to be at hand during these combustion analyses.

The equation of combustion is:

$$CH_4 + 2O_2 = CO_2 + 2H_2O.$$

As the contraction and the carbon dioxide formed cannot be measured separately owing to loss of CO_2 by diffusion into the water of the funnel, combustion is followed immediately by absorption of CO_2. One-third × (contraction + carbon dioxide) = methane.

Series no. 13. 10. ii. 19.

Macro-analysis 10. ii. 19: 92·9 % methane,
7·1 „ nitrogen.

Micro-analyses:

A. A small sample is measured off and a larger amount of atmospheric air freed from CO_2[1] is added afterwards.

1.	92·9 % CH_4	T 13·8	11·3	corrected	11·0	equal to 10·2 mm. pure methane = 6·1 % of the mixture
	+ atmospheric air	T 13·8	167·0	„	166·7	$30{\cdot}7 \times \frac{1}{3} = 10{\cdot}2$ mm. = 6·1%
	Combustion + CO_2	T 13·9	136·3	„	136·0	
	Alkaline pyrogallate	T 14·0	125·4	„	125·0	11·0 mm. oxygen left after combustion
2.	Calculated content	12·3 mm. CH_4 = 7·3 %				
	Analytical result	12·0 mm. CH_4 = 7·2				
3.	Calculated content	11·5 mm. CH_4 = 6·8				
	Analytical result	11·3 mm. CH_4 = 6·7				

B. A larger amount of pure oxygen is measured off and a smaller sample added afterwards.

4.	Pure oxygen	T 14·0	142·5	corrected	142·3	$12{\cdot}7 = 11{\cdot}8$ mm. $CH_4 = 7{\cdot}6$ %
	+ 92·9 % CH_4	T 14·1	155·2	„	154·9	
	Combustion + CO_2	T 14·2	120·1	„	119·7	$35{\cdot}2 \times \frac{1}{3} = 11{\cdot}7$ mm. CH_4 = 7·6 %
5.	Calculated content	23·6 mm. CH_4 = 14·8 %				
	Analytical result	23·2 mm. CH_4 = 14·6				
6.	Calculated content	16·2 mm. CH_4 = 9·7				
	Analytical result	16·0 mm. CH_4 = 9·6				

As it appears from the methane volumes re-calculated into per cent. the errors lie between 0 and + 0·1 %; in a single case they amount to + 0·2 %. Consequently it may be taken for granted that there is practically no systematic error in analyses of methane mixtures of 1–10 %. Mixtures containing 11–15 % may have a systematic error lying between + 0·1 and + 0·2 %.

Fractional Combustion of Gas-mixtures, containing both Hydrogen and Methane.

The fact that a heated platinum wire which is not visibly glowing causes combustion of hydrogen while on the contrary a wire of a bright red or white heat is required for combustion of methane led to the question whether it

[1] Carbon dioxide-free atmospheric air is always at hand in the potash flask.

might be possible to estimate both gases by fractional combustion. The results obtained answered our expectations.

The technique is quite the same as in ordinary combustion analyses, the only factor to be considered being, that the combustion of hydrogen takes place at the lowest possible heat by means of a "dark" wire. At the very moment the combustion occurs causing the wire to glow momentarily the current has to be broken off by a key. If the current is not interrupted instantaneously at this moment a trace of methane will also be burned.

Whether higher percentages of hydrogen can be estimated together with methane without causing a smaller or larger systematic error upon the methane percentage is a question open to future experiments. Dilution of the sample by means of an ample amount of atmospheric air will no doubt be the simplest procedure in such cases.

The methane for these analyses was obtained by means of evacuated receivers with connecting tubes and thin steel needles from the intact paunches of freshly killed cows at a slaughter-house. The gas thus collected was freed from carbon dioxide by passage through a Pettenkofer's absorbing tube filled with potash. A micro-analysis of the gas thus obtained showed a content of 52·7 % methane.

The gas-mixture employed for the series below was prepared by adding roughly estimated parts of this methane mixture, pure hydrogen, pure oxygen and pure nitrogen.

Series no. 14. 11. vi. 19.

Percentage of

H_2: 5·3, 5·3, 5·5, 5·3, 5·6, 5·4. Mean: 5·40.

σ: ± 0·13, σ_m: ± 0·05. Systematic error: + 0·05 ± 0·05. Macro-analysis 11. vi. 19: 5·45 % H_2.

CH_4: 9·3, 9·3, 9·3, 9·4, 9·2, 9·3. Mean: 9·30.

σ: ± 0·06, σ_m: ± 0·03. Systematic error: + 0·23 ± 0·03; Macro-analysis 11. vi. 19: 9·53% CH_4, 4·12 % O_2 left after combustion.

It will be seen that the methane is left totally intact by the hydrogen combustion when the directions given are followed.

A Micro-Receiver for small Gas Samples.

The quantities of gases met with in nature during biological work are often so scanty that they are lost when taken into the ordinary gas sampling tubes. Krogh has for these reasons mounted the analysis apparatus in a transport-box, serving at the same time as a stand, so that the apparatus is handy for use in nature, on board a ship or elsewhere. The small air-bubbles are then led directly into the absorption funnel of the apparatus and analysed at once. This may be practical in many cases, when only estimations of carbon dioxide and especially oxygen are wanted, but it will hardly be possible to obtain any accuracy when the combustible gases have also to be determined by the technique described in this paper. This condition led to the construction of a suitable micro-receiver for collecting and storing small gas samples during transport until analysis can take place in the laboratory.

The essential point of this simple little apparatus, making it differ from the ordinary receivers in use, is the circumstance that it has no stopcocks, the gas being forced to bubble in through a column of mercury. The sample is thus locked up between two columns of mercury. The construction and use will readily be understood from Fig. 1, *B*. The receivers are easily made by means of a blow-pipe. The dimensions are: diameter of glass tubing 6 mm.; length of longer limb 70 mm., 25 of which are drawn out for connection with

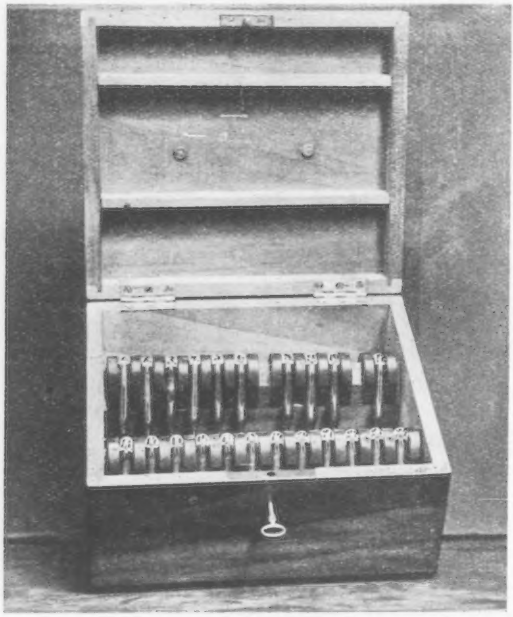

Fig. 2. Transport-box for 24 micro-receivers. The cover contributes to fix the receivers during transport
[*About ¼ actual size.*]

a narrow rubber tubing; length of shorter limb 50 mm., being equally drawn out. The angle between the two limbs measures 70–80°. Narrow india-rubber tubing, which is not apt to stick together, is used for connection. The rubber tubings are closed by means of small stiff clips, made of spring steel. A micro-receiver of these dimensions may hold about 1·7 cm.^{3} gas at ordinary pressure locked up between two mercury columns 3 cm. in height. As all contaminating air is driven out of the connecting tubing and cannula by means of mercury, even smaller air-bubbles may be taken over into the absorption funnel without loss.

A transport-box for 24 micro-receivers will be seen in Fig. 2.

As it could not be excluded beforehand that a minute diffusion might take place along the sides of the tube past the mercury, which is known not to adhere to glass like most fluids, the following control experiments were carried out.

The mercury columns serving for locking up the samples measured on an average 3 cm. in height in all three experiments. A certain pressure was established in all the receivers before they were clipped.

Experiment no. 1. Tightness test for carbon dioxide.

Micro-analysis of gas-mixture before filling of micro-receiver (series no. 10):

21·90 % CO_2

„ after 15 days (1) 21·2 „

(2) 21·3 „

The loss has been about 0·65 % CO_2 in the course of 15 days.

A calculation of the loss per 10 % in 24 hours makes it about 0·02 %.

Experiment no. 2. Tightness test for carbon dioxide.

Micro-analysis of gas-mixture before filling of micro-receiver (series no. 11):

32·9 % CO_2

„ after 4 × 24 hours (1) 32·4 „

(2) 32·2 „

(3) 32·3 „

The loss has been about 0·65 % carbon dioxide in the course of 96 hours.

A calculation of the loss per 10 % in 24 hours makes it about 0·05 %.

Experiment no. 3. Tightness test for hydrogen.

Micro-analysis of gas-mixture before filling of micro-receivers *A* and *B*:

(1) 11·9, (2) 12·0, (3) 12·0 % H_2.

Micro-analysis of gas-mixture in receivers *A* and *B* after 24 hours:

A (1) 12·0, (2) 12·0 %,

B (1) 12·1, (2) 12·0 %.

No loss of hydrogen has taken place in the course of 24 hours.

Discussion of results. The issue of experiment no. 3 ensures the absolute tightness of the receivers even for hydrogen within 24 hours. The losses of carbon dioxide in 24 hours would as seen by the calculations of the results of the two first experiments be quite insignificant, the high percentages and the exceedingly long time being taken into consideration. It may also be possible that no diffusion has taken place at all, the losses being due to unnoticed impurities of the mercury.

The conclusion drawn from these preliminary experiments is, that the micro-receiver described is absolutely reliable within 24 hours. Gas samples ought consequently when the utmost accuracy attainable is wanted to be analysed before this time has elapsed.

SUMMARY.

1. The micro-analysis apparatus described by Krogh is also applicable to hydrogen and carbon monoxide estimations, a solution of colloidal palladium plus sodium picrate and an acid cuprous chloride solution serving respectively as absorbing agents.

2. A modification of this apparatus with combustion funnel is described. It is possible by means of this apparatus to estimate CO_2 and O_2 by absorption, H_2 and CH_4 by combustion in a given air-sample of about 80 mm.^{3}—the size of a small pea. CO may be estimated either by absorption or combustion according to circumstances.

N_2 (+ inert gases) is calculated as residue.

3. Fractional combustion of hydrogen and methane appearing together is practicable by means of this technique.

4. The accidental error (standard deviation) in the present micro-analyses corresponds on an average to an error of reading of $\pm$ 0·1 mm.

5. The systematic error differs for the different gases. While being without any practical importance in estimations of O_2, H_2, CH_4 and probably also CO, it has to be considered in carbon dioxide analyses.

6. A micro-receiver for small gas samples is described.

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II. ON THE DETERIORATION OF COTTON ON WET STORAGE.

By NANCY FLEMING and AAGE CHRISTIAN THAYSEN.

(*Received December 4th, 1919.*)

(With Plate I.)

It is a well-known fact that exposure to damp renders cotton extremely brittle and short-fibred, and increases its alkali solubility. The extent of this damage is often very considerable, may in exceptional cases cause the complete destruction of the cotton affected, and will often entail the loss of from 10–15 % of the cotton in the form of exceptionally short fibres, the so-called "fly."

In a preliminary report to the Admiralty, dated 19/3/19, an account was given of some experiments carried out in connection with the investigation of this deterioration on wet storage. The conclusion arrived at was, that cotton does not deteriorate on wet storage in the absence of micro-organisms, so that bacteria, in the wider sense of this word, must be considered responsible for the breakdown of the fibres. It was furthermore found that the activity of the bacteria can be brought to a standstill, and the deterioration prevented, if the moisture content of the cotton is reduced to below 9 %.

In view of the commercial importance of this breakdown of the cotton fibres, it was thought desirable to extend the investigation and to examine the types of micro-organisms responsible for the damage; secondly, to establish the source of the infection and the changes taking place in the structure of the fibres by the action of the bacteria, and finally, to determine to what extent this damage to the fibres is noticeable in the various types of cotton.

The present paper deals with some of the results obtained in the investigation of the changes caused by bacteria to the structure of the cotton fibre, and with the method worked out for the quantitative determination of this type of deterioration of cotton.

An investigation of the changes in structure of the attacked fibres was not found practicable by direct microscopical examination, as they were too minute to be examined carefully, even by the oil immersion lens. It was decided, therefore, to make use of Cross and Bevan's viscose process for the treatment of the fibres before the microscopic examination. The viscose process has already been used by Balls [1918] for the demonstration of the daily layers of growth in the cell wall of cotton hairs. When applied as suggested by this author, it causes the cotton fibre to swell to about five times its normal size.

The result of the application of the viscose process to the damaged cotton fibres proved most instructive. Some of the observations made will be outlined in the following pages.

The technique of the method, as applied for our purpose, was substantially the same as that used by Balls, except that, when using 15 % Na(OH), it was found possible to dispense with the application of alkali *in vacuo*.

In our investigation we proceeded as follows. From 0·1 to 0·3 gram of cotton of the sample to be examined was boiled for a short time in a 1 % Na(OH) solution, then washed in running water, dipped in a 1 % acetic acid solution, and again washed in water. After removal of most of the adhering water the cotton was transferred to a small stoppered glass bottle with a wide opening and 1·5 cc. of 15 % Na(OH) solution and 1·5 cc. of carbon disulphide were added. In this mixture the fibres were left for 15–45 minutes, then placed on an ordinary glass slide, covered with a cover slip, and a drop of tap water left to diffuse under the latter. The preparation was now ready for microscopic examination.

Fig. 1, Pl. I, shows the behaviour of normal fibres after the viscose treatment. The daily growth rings, described by Balls, are very marked. Particularly characteristic in most of the fibres is their prevalent beaded appearance. This, no doubt, is brought about by the cuticle giving way to the internal pressure created by the rapidly swelling cellulose. The cuticle can in many cases be seen rolled up between two individual beads as a narrow ring of high refractive power. In other fibres the cuticle seems to have burst longitudinally, and in this case forms a corkscrew band round the swollen cellulose.

If an attacked fibre is treated by the viscose process, quite a different effect is obtained, which varies with the type of micro-organism responsible for the deterioration.

Figs. 2 and 3 are from photographs taken from attacked fibres after treatment. In Fig. 2 a streptothrix was responsible for the action, while a cellulose-decomposing schizomycete caused the breakdown of the fibre in Fig. 3.

It will be noticed that in Fig. 2 the cuticle, and most of the cellulose, has been perforated in numerous places. The cuticle has apparently in this case lost its power of resistance, and the cellulose swells uniformly. No beads are observed.

The same can be said of the fibres in Fig. 3, where the swelling, as already mentioned, is due to a cellulose-decomposing schizomycete. The cuticle of the fibres in this specimen seems to have been eaten away over wide areas. It looks as if the destruction in this case was progressing uniformly from the surface toward the interior.

Fig. 2 explains the appearance of the large number of short fibres found in deteriorated cottons. They are produced by the action of a streptothrix, a form of micro-organism which is always found in large numbers in weathered cottons.

The damage of the true schizomycete is due, less to the formation of fly, than to a general weakening of the fibre.

The fibres, from which Figs. 2 and 3 were taken, had been exposed to the action of micro-organisms for a considerable time—over a month. It appeared of interest, therefore, to determine how soon after exposure to dampness, the action of the micro-organisms would be noticeable on the fibres. Samples of various grades of cotton were, therefore, wetted with tap water and left at ordinary room temperature. It was found, that a cotton waste sample with a bacterial content of about 1 million per gram, showed signs of extensive damage after five days' exposure. On the other hand, a carefully picked sample, taken direct from the bolls, and with only 20,000 micro-organisms per gram, could be left damp for twenty days before the fibres were visibly attacked. If, however, such samples were infected with the characteristic micro-organisms, the damage was considerably accelerated.

Fig. 4 shows a sample of ordinary cotton waste which has been exposed to dampness for five days. It will be noticed that very few of the fibres have any of their cuticle left intact.

Another point of interest is the difference between the ways in which the streptothrix and the true bacteria attack the fibres. The two types of damage are so markedly different, that it is almost possible by the microscopic examination to indicate where the fibres were damaged by a streptothrix, and where by bacteria.

These remarkable changes, found to occur in the structure of the cotton fibre after exposure to dampness, made it natural, on this basis, to attempt the elaboration of a method for the quantitative determination of damaged fibres in good cotton.

Most raw cottons, of American and Indian origin, have at one time or other been exposed to rain or excessive dampness, and others may have been deliberately wetted. In both cases the result is a damage to the fibres, which in its earlier stages, cannot at present, be detected. It is true, that where the moisture content of the cotton remains high—over 9 %—a determination of the amount of water present will be a useful indication of damage; but, in many cases, where the wetting occurred on the farms or after ginning, the cotton is likely to dry up when taken to other districts, for instance for transport to Europe. Moreover the determination of the moisture content can only be an indication, and not a quantitative method by which the cotton merchant is able to determine how far the fibres of a particular cotton have been damaged, and how much of it he is likely to lose during its subsequent treatment at the mills.

The alkali solubility test would, no doubt, in many cases of excessive damage, give good results; but where it is a case of detecting 10, or 5, or perhaps even 2 % of damaged fibres, this method is not sufficiently sensitive. In a later paper it is hoped to substantiate this assumption.

The method for the detection and quantitative determination of damaged fibres in cotton by microscopic examination, as worked out on the basis of the above observations has, so far as it has yet been tried, given very satis-

factory results. It may be of interest to give a few examples of the results obtained.

A sample of damaged cotton, in which all fibres were found to be attacked, was carefully mixed with various quantities of good cotton, picked direct from the boll, and containing normal fibres only. A sample of this mixture containing 30 % of damaged fibres was, on examination, found to have 29·6 %. A second determination of the same sample gave 29·5 %, a third 27·3 %. A mixture made up to contain 10 % was found to have 8·9 %; a 5 % sample 5·4 %, a 4 % sample 4·6 %, and a 2 % sample 2·0 % and 2·5 %.

The broad outlines of the method as now in use[1] are as follows. The sample to be examined is treated with the carbon disulphide and alkali mixture; the fibres are then spread out on a slide, as already described, and counted under the microscope. From each sample three preparations are made. The average of 10 counts from each preparation gives the required figure.

Conclusions.

In cotton which has been exposed to dampness the action of micro-organisms materially alters the structure of the fibres, giving rise to the formation of exceptionally short fibres, the so-called "fly."

The extent of this damage can be quantitatively determined by a method outlined in this paper.

REFERENCE.

Balls (1918). The existence of daily layers of growth in the cell wall of cotton hairs. Author's MS.

[1] The details of the technique will be published later.

Fig. 1. ×45.

Fig. 2. ×45.

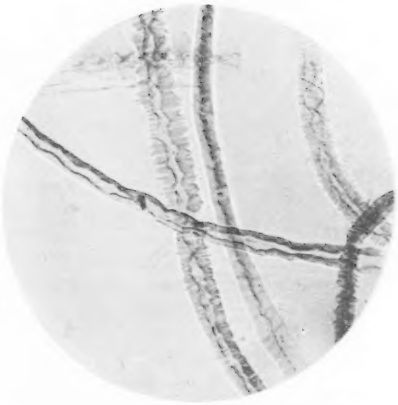


Fig. 3. ×45.

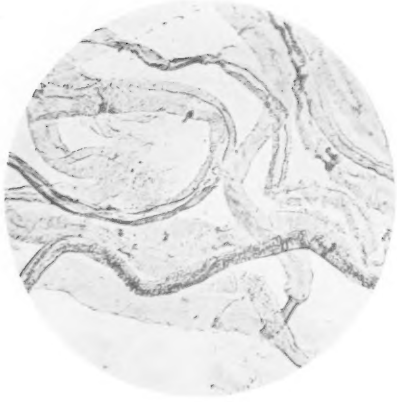


Fig. 4. ×45.

III. ADSORPTIVE STRATIFICATION IN GELS, III.

By SAMUEL CLEMENT BRADFORD.

(*Received December 11th, 1919.*)

(With Plate II.)

The first record of the formation of noticeable structures by the diffusion of precipitating reagents appears to have been made by Runge [1855], who experimented with a number of pairs of inorganic reagents in blotting paper. In 1874 Monnier and Vesque observed the production of large crystals by slow diffusion through strips of filter paper and gels respectively. Three years later Ord allowed ammonium oxalate to diffuse through an isinglass gel in an open tube immersed in calcium chloride solution. The precipitate consisted first of growth forms changing into spherites towards the calcium chloride. The diffusion of solutions into gels was studied extensively by Pringsheim [1895] in 1890 and 1892. The results of his experiments were communicated to the Berlin Academy in 1891 and 1892, but not published until 1895. Pringsheim allowed two solutions, capable of interacting with the formation of a precipitate, to diffuse in opposite directions through gelatin gels contained in the horizontal portion of a graduated U-tube of special form. The rates of diffusion for different concentrations and various reagents were observed, and the important law discovered that, after the meeting of the reagents, diffusion took place only from the hypertonic into the hypotonic reagent. From the number of his experiments it would be surprising if Pringsheim had not observed cases of band formation, especially as U-tubes favour this result. However, such concentrated solutions were employed, from $N/2$ to $12N$, as usually to prevent stratification of the precipitate. Nevertheless examination of his illustrations shows the formation of two bands in several cases. As a botanist he refers to the occurrence: "In many cases not one but two separated precipitates are produced, which may perhaps be due to dissociation phenomena."

The first observation of a series of layers must be ascribed to Lupton [1892]. Unfortunately Lupton did not investigate the phenomenon, and the nature of his bands is left to conjecture. But in 1896 Liesegang [1896], continuing Pringsheim's experiments, obtained the remarkable series of silver dichromate rings now well known. The original experiment was made with a drop of silver nitrate on a glass plate coated with 5 per cent. gelatin containing a small quantity of potassium dichromate. Liesegang rightly perceived the

importance of the phenomenon and commenced an experimental study, the first part of which was published [1898] in a pamphlet which has not been available in London until recently. In these researches he used mostly ammonium dichromate in gelatin gels contained in glass tubes inverted in aqueous silver nitrate. He showed that the spaces between the layers are completely free from unchanged ammonium dichromate, since silver nitrate poured over a plate preparation produced no further effect. Plates examined with X-rays showed dark shadows, corresponding to the A-lines as he called them, with the spaces almost clear. "There is extraordinarily much more silver in the A-lines than in the spaces."

Liesegang also obtained "essentially similar structures" with other insoluble precipitates. Ammonium chromate gave rings almost exactly like those of the dichromate. Using lead nitrate, instead of the silver salt, the bands were wholly like the silver dichromate ones, but not so sharp, and the spaces were filled with a cloudiness due to yellow lead chromate. A-lines were also obtained with lead iodide and thiosulphate, the latter giving sharp white lines separated by glass clear spaces, and with silver ferric oxalate and Prussian blue. Liesegang perceived that wider study of the phenomena might enable biologists artificially to imitate the structures of living organisms. He suggested that banded agates might have arisen in this way and warned microscopists that their methods of staining might lead to the production of fresh structures in the objects under examination.

After having inspected Liesegang's "astonishing" preparations, Ostwald interpreted the A-lines as due to supersaturation, remarking that "Precipitates which appear in the colloidal form give no A-lines, neither do those which are ordinarily (i.e. visibly) crystalline; whatever is the reason in such cases has not yet been discovered" [1897][1]. The posthumous theory of the late Lord Rayleigh [1919] is essentially similar.

The supersaturation theory was subjected to mathematical analysis by Morse and Pierce [1903]. Working with potassium chromate in the gel, the reaction takes place in accordance with the relation:

$$2AgNO_3 + K_2CrO_4 \rightleftarrows Ag_2CrO_4 + 2KNO_3.$$

For equilibrium in the presence of the solid phase the concentrations must satisfy the relation:

$$Ag^{\cdot 2} \times CrO_4'' = k, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where k is the solubility product. Morse and Pierce inquired whether there is a relation in the case of a supersaturated solution similar to (1), with, however, a different constant, that defines the limit of supersaturation, e.g.

$$Ag^{\cdot 2} \times CrO_4'' = H. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

Assuming that the formation of a precipitate has no influence on the progress

[1] It has previously been shown, Bradford [1917], that the adsorption theory explains both these facts.

of the diffusion, by the application of Fick's law, they were able to obtain the formula:

$$H = \frac{4U_0^2V_0}{\pi^{\frac{3}{2}}}\left[\int_{\frac{x}{2a\sqrt{t}}}^{\infty} e^{-\beta^2}d\beta\right]^2 \cdot \int_{-\frac{x}{2b\sqrt{t}}}^{\infty} e^{-\beta^2}d\beta, \quad \text{.....(3)}$$

where x is the distance of a band from the surface of the gel, t the time from the commencement of the experiment, U_0 and V_0 are the original concentrations of the reaction components, a^2 and b^2 their diffusion constants, and β is merely a constant of integration.

From this Morse and Pierce deduced that $\frac{x}{\sqrt{t}}$ must be constant, which they were able to verify, that the change of concentration of the solution in the gel can be neglected and that:

$$H = 1{\cdot}4 \times 10^{-6}\left(\frac{\text{mol.}}{\text{litre}}\right)^3$$

and

$$a^2 = 1{\cdot}54\left(\frac{\text{cm}^2}{\text{day}}\right).$$

This indicates that, with N $AgNO_3$ and $N/75$ K_2CrO_4, precipitation occurs at each new band when the concentration of the silver ion is about $N/200$, that of the chromate being unaltered at $N/75$. Moreover, since the ordinary solubility product of silver chromate is $5{\cdot}1 \times 10^{-13}$, the supersaturated gelatin at the concentration of precipitation held in solution $\frac{\sqrt[3]{1{\cdot}4 \times 10^{-6}}}{\sqrt[3]{5{\cdot}1 \times 10^{-3}}}$ or 140 times the amount of silver chromate required to saturate it in the presence of the solid phase. It will also be noted that the figure, 1·54, found for the diffusion constant of silver nitrate in gelatin is much greater than that in pure water, 1·13 to 1·20, determined by Kawalki. These deductions are very remarkable and it will be endeavoured to show that they are contrary to fact. Morse and Pierce also obtained bands in gelatin with lead sulphate; silver oxalate, carbonate, phosphate, thiocyanate and bromide; cobalt hydroxide; barium oxalate; mercurous bromide and carbon dioxide. They also found that bands were produced in capillary tubes in aqueous solution, which broke down after the formation of two or three.

The supersaturation theory has been criticised by a number of writers. Bechhold [1905] considered that, in addition to supersaturation, the production of banded precipitates was influenced by the solubility of the precipitate in one or other of the salts present or formed by the reaction, and by the coagulating or inhibiting effect of ions, as in the case of suspensions and colloid solutions. By mixing 1 cc. of silver nitrate solutions of different strengths with 3 cc. of $M/100$ $(NH_4)_2Cr_2O_7$ in 2·5 per cent. gelatin sols, Bechhold found that deep turbidity was produced with a concentration as low as $M/533$ of $AgNO_3$.

The metastable product can be calculated from this value:

$$Ag^{\cdot 2} \times Cr_2O_7'' = \left[\frac{1{\cdot}88 \times 10^{-3}}{4}\right]^2 \cdot \left[\frac{3 \times 10^{-2}}{4}\right] = 1{\cdot}64 \times 10^{-9}.$$

It will be seen that although silver dichromate is more than twice as soluble .

as the chromate, yet the metastable product calculated from Bechhold's figures is very much less than that deduced from the supersaturation theory for the latter substance by Morse and Pierce.

Since nitrates tend to liquefy the gelatin, Bechhold thought they should favour the formation of a precipitate, and this would be the effect of the ammonium nitrate formed in the reaction. Similar experiments were therefore made both with $N/160$ and $N/320$ $AgNO_3$ mixed with $M/100$ $(NH_4)_2Cr_2O_7$ in 5 per cent. gelatin containing increasing amounts of $(NH_4)NO_3$. The turbidity increased to a maximum, diminished and again increased. Parallel experiments in water without gelatin showed similar maxima and minima, though somewhat less definite. These latter results are particularly interesting as they suggest that ordinary supersaturated solutions are affected by ions in the same way as sols. The question of the relation of sols to supersaturated solutions is in urgent need of further study, which would probably throw much light on the nature of solution.

Bechhold realised that, if the ammonium nitrate formed by the reaction plays a part in the ring formation, an increase in the concentration of this salt must cause a change in the ring structure. The bottom of a glass dish was covered half with a layer of 5 per cent. gelatin containing $M/20$ $(NH_4)_2Cr_2O_7$ and half with chromated gelatin with addition of $N/4$ $(NH_4)NO_3$. A drop of silver nitrate was placed over the division. The rings in the ammonium nitrate half were appreciably wider than in the other half and very slightly closer, so that separation of the bands was not apparent till a greater distance from the centre. This shows that the formation of the bands is undoubtedly affected by the presence of soluble salts in the gel. However the concentration of the $(NH_4)NO_3$ was six times greater than would be produced in an ordinary experiment. Similar ideas, though without further direct experimental support, were suggested later. In a lecture at the Royal Institution in 1912, Sir J. J. Thomson supposed that the soluble substances produced by the reaction might act in a similar way to certain bodies known as stabilisers which prevent the precipitation of colloidal solutions, and Freundlich and Schucht [1913] thought that the formation of a precipitate might be due to autocatalytic coagulation by electrolytes.

It seemed desirable to add increments of by-product more comparable with the concentration in which they are usually produced. Tubes containing (1) 20 cc. of $N/20$ Na_2CO_3 agar gel and (2) the same with $N/20$ NaCl were treated with 5 cc. of dilute $CaCl_2$ solution. Diffusion proceeded at the same rate in each tube with the formation of bands at distances below the surface of the gel in mm. as follows:

(1)	20·5	22·5	25	28	32·5	35·5
(2)	20·5	22·5	24·5	—	33	35·5

In another experiment 15 cc. of $N/5$ K_2CrO_4 solution was poured on (3) 15 cc. $N/200$ $Pb(NO_3)_2$ agar gel, and (4) the same gel with addition of KNO_3 to $N/100$.

The tubes developed beautiful series of clear-cut strata of lead chromate very close together, the average distance of the last twenty layers being respectively 0·70 and 0·67 mm. Comparison with Bechhold's result shows that any influence of the by-product of the reaction is to reduce the distance between the strata, i.e. to inhibit separation of the bands, but such effect is practically negligible.

Hatschek [1911, 1, 2; 1912] used a number of fresh compounds and studied for the first time reactions in various gels, including the inorganic gel of silicic acid. He showed that precipitates tend to form larger crystals in gels than in aqueous solution and that the particles of banded precipitates are usually microscopic spherical aggregates, and was able [Hatschek and Simon, 1912] to explain by his results many features of the occurrence of gold in quartz. He [1914] showed that the supersaturation theory is inadequate to explain the complicated phenomena of stratified precipitation. If, as Ostwald thought, the silver dichromate is deposited on the ring from the neighbouring region which is supersaturated in regard to this salt, it should be impossible to produce a second set of rings in a tube already containing one, or to obtain a stratified precipitate in a gel previously sown with crystals of the insoluble precipitate. Nevertheless he obtained perfect stratifications of lead iodide in agar containing suspended lead iodide [1912] and his pupil, de Whalley, succeeded in producing a second system of lead chromate bands in agar, superposed on a finer system of strata of the same substance [Hatschek, 1914]. These experiments appear to show that bands can form although supersaturation is prevented.

Liesegang [1914] showed that strata of silver dichromate do not form in gelatin which has been purified from mineral matter by soaking in slowly changing water. It is necessary to add gelatose and a small definite amount of (citric) acid for each particular concentration of soluble dichromate in the gel. The bands are however thicker and more widely spaced than in the unwashed gelatin. Further addition of acid yields a continuous mass of colloidal silver dichromate. Moreover it has been found that neither silver chromate nor dichromate forms bands in agar gel. Indeed the nature of the gel appears to have a very remarkable influence on the form of the precipitate in all cases. This was first insisted upon by Hatschek as a necessary consideration for a complete theory of the phenomenon. Hatschek's view of the inadequacy of the supersaturation hypothesis was endorsed by Wo. Ostwald [1917].

Experimental study reveals many minor points for which it is difficult, or impossible, to account on Ostwald's hypothesis. Moreover it seems particularly hard to devise a direct proof of the supersaturation theory. The only experimental evidence so far adduced in its favour appears to be the verification by Morse and Pierce of the constancy of the ratio of the distance of a band from the surface of the gel and the square root of the time of formation. This is by no means a direct proof of the theory. Moreover, as already pointed out, the results deduced at the same time for the metastable product and the diffusion

constant of silver nitrate, as well as the non-diffusion of hypotonic reagent are remarkable. Indeed the latter is at once seen to be incorrect by working with a highly-coloured solute in the gel.

It appeared to be a simple matter to make a direct test of the value of the metastable product which could be compared with that required by the supersaturation theory. 5 per cent. gelatin gels of Nelson's "crystal" leaf gelatin were prepared containing decreasing amounts respectively of potassium dichromate and chromate. These were treated with dilute solutions of silver nitrate of suitable concentration. The silver nitrate was always hypertonic, so that, by Pringsheim's law, the precipitate should always be formed in the gel.

The value found by Morse and Pierce for silver chromate was $H = 1{\cdot}4 \times 10^{-6}$. Since the solubility of silver dichromate is $0{\cdot}0_3 19$ mols per litre, as compared with $0{\cdot}0_4 8$ mols per litre for the chromate, the value of the metastable product for silver dichromate, which would correspond to that found by Morse and Pierce for the chromate, would be $\frac{1{\cdot}4 \times 10^{-6}}{5{\cdot}1 \times 10^{-13}} \times (1{\cdot}9 \times 10^{-4})^3 = 1{\cdot}9 \times 10^{-5}$. Below these values of H no precipitate should be formed. Yet it has been seen that Bechhold obtained a deep turbidity in 2·5 per cent. gelatin sols with concentrations of dichromate corresponding to $H = 1{\cdot}64 \times 10^{-9}$. Since reactions in gelatin gels must take place in the liquid dispersion medium [Bradford, 1918] which contains only a few tenths of one per cent. of gelatin, the protective effect of the gels could not be greater than in the sol state. The experiments in gels are tabulated below:

Table I.

$K_2Cr_2O_7$	$AgNO_3$	H	
$M/200$	$M/50$	$2{\cdot}0 \times 10^{-6}$	Red surface precipitate in flakes, faint bands at 1·6, 2·3 mm. Then beautiful black spherites of $Ag_2Cr_2O_7$, up to 0·17 mm. diam., to depth of 1·5 cm.
$M/250$	$M/50$	$1{\cdot}6 \times 10^{-6}$	Red surface layer in flakes, faint bands at 1·3, and 3·6 mm. A few spherites below
$M/300$	$M/50$	$1{\cdot}33 \times 10^{-6}$	Red dusty ppt. above, faint layers at 0·8 and 3 mm.
$M/350$	$M/50$	$1{\cdot}1 \times 10^{-6}$	Faint dusty layer above, very faint layer at 0·4 mm.
$M/300$	$M/100$	$1{\cdot}6 \times 10^{-7}$	Fine dusty ppt. on surface. Ochre coloration below.
$M/400$	$M/100$	$1{\cdot}25 \times 10^{-7}$	Dusty red ppt. on surface. Ochre coloration below.
$M/500$	$M/100$	$1{\cdot}0 \times 10^{-7}$	No distinct ppt. on surface. Ochre coloration.
$M/1000$	$M/100$	$5{\cdot}0 \times 10^{-8}$	No distinct ppt. on surface. A few black spherites of ? $Ag_2Cr_2O_7$, 0·03 mm. in diam. to depth of 4 or 5 cm. below surface.

Table II.

K_2CrO_4	$AgNO_3$	H	
$M/200$	$M/70$	$1{\cdot}0 \times 10^{-6}$	Beautiful series of bands at 0·0, 0·4, 0·8, 1·3, 1·9, 2·3, 3·1*, 4·7*, 6·8*. Clusters of spherites at 7·0 and 9·0 mm., largest 0·02 mm. diam.
$M/400$	$M/70$	$5{\cdot}0 \times 10^{-7}$	Thin red layers at 0·0, 0·6, 1·6, 2·5, 4·0, broken at 5·2 mm. Spherites down to 12·4 mm., largest 0·01 mm. diam.
$M/800$	$M/70$	$2{\cdot}5 \times 10^{-7}$	Red surface layer with faint band in ochre opalescence extending to 1·5 cm.
$M/400$	$M/140$	$1{\cdot}25 \times 10^{-7}$	Fine red ppt. on surface.
$M/800$	$M/140$	$6{\cdot}25 \times 10^{-8}$	Fine ppt. on surface.
$M/800$	$M/210$	$2{\cdot}75 \times 10^{-8}$	Faint ppt. on surface.

* Layers composed of spherites.

It will be seen that with $H = 1{\cdot}0 \times 10^{-6}$, which is already less than Morse and Pierce's figure, silver chromate still forms a beautiful series of bands. The concentration at the bottom of the tube must be very much less than this value. In all the experiments there was some subsidiary white precipitate, as always occurs with silver nitrate in gelatin. Actually, with very low values of H, the amount of white precipitate was much greater than that of the chromate, so that the effective value of H for the silver chromate must have been considerably less than that corresponding to the concentrations used. As the ionic product becomes smaller, the density of the precipitate in the surface layer of the gel becomes less until, even under the microscope, it is difficult to determine whether the precipitate is formed in the gel or in the solution. Pringsheim's law indicates that the precipitate is always in the gel. But, without making any assumption in regard to this, it is certain that silver dichromate and chromate respectively form precipitates with values of H lower than $1{\cdot}1 \times 10^{-6}$ and $2{\cdot}5 \times 10^{-7}$, corresponding to the appearance of a layer below the surface. These are respectively 16 and 6 times less than indicated by the supersaturation theory[1]. These experiments are sufficient to invalidate the assumption made by Morse and Pierce, that the formation of a precipitate does not affect the head under which diffusion takes place. That is to say the precipitate does affect the course of the diffusion.

Consideration of the facts (*a*) that the hypotonic reagent is removed from the spaces between the layers and (*b*) that only precipitates which are either colloidal or extremely finely divided separate into strata, leads naturally to the conclusion that it is the adsorbing action of the large surface of the precipitate which removes the solute from the layer of gel in its vicinity, so that the hypertonic reagent, diffusing through, has to traverse an exhausted zone of gel before meeting with sufficient solute to produce a new band.

Microscopic examination of the precipitates which occur in banded form usually shows that they consist of spherites. In the case of silver chromate and dichromate in gelatin, and manganese sulphide in agar, the individual particles are generally too small for their nature to be determined. In the majority of cases the beautiful spherical particles are easily visible, sometimes, as in the case of copper carbonate in agar and calcite in silicic acid, the spherites are large enough to show distinct banding and give a dark cross and colour rings in polarised light. The origin and structure of spherites has been discussed as far as seemed possible at present in a previous paper [Bradford, 1918]. Further work is in progress. For the purpose in view it is sufficient to recall that these apparently globular crystals actually consist of a great many radial needles, so that small spherites have an enormous specific surface and may be regarded as being in the colloidal state. A few precipitates, such as calcite in silicic acid gel, appear first, in the more concentrated regions, as tiny perfect rhombohedra, which become more aggregated, as the velocity of crystallisa-

[1] These figures receive confirmation from some experiments to be published shortly.

tion diminishes with dilution of the reagents, and finally occur as perfect spherites of macroscopic size.

In all the cases examined, therefore, banded precipitates have very large specific surface and may be expected to behave in the same way as finely divided solids in adsorbing dissolved substances from their vicinity. This effect occurs in the filtering of solutions through fine powders, where the dissolved particles are retained by the solid. It is well illustrated by Dreaper and Davis' night-blue experiment.

The marked adsorption of solutes containing like ions by sparingly soluble solids is generally recognised, and may well be akin to the remarkable attraction of nutrient material from solution by growing crystals, both being probably of electrical origin. Indeed the adsorption of solute in the formation of banded precipitates may be looked upon as comparable with the mechanism of crystal growth. Consequently it can hardly be doubted that precipitates, formed in media which can hold them in place, will tend to remove the soluble reaction components from their vicinity, and this effect will be the more marked the greater the specific surface of the precipitate. Moreover, since the reagent diffusing into the gel is always in excess, any of the solute in the gel, which is adsorbed, will be precipitated with the formation of fresh surface. The adsorbing surface is, therefore, always "clean" with regard to the solute in the gel.

As the adsorption progresses, solute will diffuse from the more concentrated region of the gel into the depleted zone, and a concentration gradient will be set up, the diffusion becoming more rapid as the concentration in the neighbourhood of the precipitate diminishes. The process will continue until the concentration of the adsorbed solute, at the surface of the precipitate, is reduced to the value corresponding to the solubility product of the solid under the particular circumstances. When this happens, the concentration of the solute in the adjoining region of gel must be very much less than this value, but will gradually increase in the direction away from the precipitate. In this manner a considerable zone of gel will be practically exhausted of solute. Any hypertonic reagent diffusing into this region will not cause the formation of a precipitate, since the ionic product will be less than the critical value. The growth of the band under consideration will then have ceased. A new layer will eventually be commenced at a distance from it, where the hypertonic reagent finds sufficient concentration of solute in the gel. In this paper the actual process of precipitation, whether through the formation of sols or supersaturated solutions, is not considered. Much further work appears to be necessary before the mechanism of precipitation in gels can be completely elucidated. It will be seen, however, that although some kind of supersaturation may, and probably usually does, occur to a greater or less degree before precipitation, this is not necessary for the formation of separate bands, and could hardly account for the occurrence of bands of very insoluble substances, such as silver chloride, which the writer has obtained several millimetres apart in agar, this gel having much less protective effect than gelatin.

It will thus be seen that the adsorption theory is almost diametrically opposed to the supersaturation theory. For whereas the latter hypothesis attributes the formation of bands to the influence of supersaturation preceding precipitation and requires that no appreciable diffusion shall occur of the solute dissolved in the gel, the former theory needs no assumption as to the mechanism of precipitation and claims exhaustion of the solute from the region of the gel in the vicinity of the precipitate.

Direct proofs of adsorption are readily forthcoming. By working with deeply coloured solutes it is possible to watch the progress of the adsorption in the neighbourhood of the precipitate. With silver chromate in gelatin the white subsidiary precipitate due to impurities makes it difficult to observe the exhaustion of the gel. But with lead chromate in agar the effect is easily seen. When viewed for the first time the phenomenon is almost astonishing. This is shown in Fig. 1, Pl. II, which is reproduced from a previous paper.

It has been objected that the adsorption theory takes no account of the influence of the gel. However, it has been shown [Bradford, 1917] that the different effects obtained with the same insoluble substance in different gels can be explained as due to the influence of the gel, or reaction medium, on the factors K and P in von Weimarn's formula, which determine the specific surface of the precipitate and therefore its adsorbing power. By the application of von Weimarn's theory it is possible to obtain silver chromate and dichromate strata in agar gel which, in absence of subsidiary precipitate, are, if possible, more beautiful than in gelatin. It is hoped to publish these experiments shortly. The same recipes give good rings in filter paper.

Both these and plate preparations in agar show the adsorption effect even more strikingly than with lead chromate. A ring of perfectly colourless gel gradually develops round each band of precipitate, the precipitate ceases to grow, and nothing can be seen except the slowly widening colourless zone. Suddenly a fresh ring commences with a deep reddish-yellow coloration at the edge of the colourless zone where it joins the yellow colour of the soluble chromate.

These experiments with coloured solutes show conclusively that the gel is actually exhausted of solute in the vicinity of the precipitate. Whether the effect be due to adsorption, or to any other cause to be determined, it is sufficient to account for the separation of the bands.

There is however little room for doubt that the colourless zone is really due to adsorption. It can easily be shown that finely divided powders actually have the power of producing a similar result. Naturally it would be inferred that the effect would be less noticeable in this case, since, without excess of hypertonic reagent, the adsorbing surface would quickly become contaminated. This is why a fresh series of bands can form upon an old one. Columns of fuller's earth were embedded in 1 per cent. agar gels above the same gels deeply coloured with various dyestuffs. Some of these showed results more intense than others. With night-blue the effect was most extraordinary.

Within about twenty minutes a perfectly colourless narrow zone could be seen below the adsorbing column. This quickly grew, and in 24 hours had reached a depth of four or five millimetres. All this time the sharpness of the colour transition was remarkable. Practically the whole of the region of gel affected was nearly colourless, the change to deep blue taking place almost suddenly. Gradually the colour gradient became less steep as the exhausted zone increased, but the effect persisted for months. Fig. 2 shows the effect after a few days. With catalpo no. 1 and magenta in 1 per cent. agar the result is equally striking and lends itself better to photography, Fig. 3 (after 17 days). From these experiments there can be no doubt that adsorption is capable of causing the extraction of soluble substances from a considerable zone of gel. Similar experiments were also performed with lead chromate as adsorbent and potassium chromate and dichromate as solutes. The concentration of potassium chromate in Fig. 1 was $N/20$, but since $N/200$ best gives bands of lead chromate, this concentration with $N/300$ and $N/100$ was used. The adsorption effect became visible almost instantaneously. It is best seen by standing with the back to the light and holding a piece of white paper sloping backwards at about 45° behind the tube. An exceedingly narrow zone of a much lighter colour immediately becomes visible beneath the column of lead chromate. The effect however increases much more slowly than with the colloidal substances previously examined. Presumably this is due to contamination of the adsorbing surface. Unfortunately also the colour of the dilute gel is not easy to photograph. Fig. 4 shows the effect with $N/300$ K_2CrO_4 after 30 days. The columns of adsorbent were made into a thin cream with water and poured on to the agar gel, care being taken to prevent the lead chromate penetrating between the gel and the tube. By using lead nitrate solution, of the same strength as the potassium chromate in the gel, instead of pure water to moisten the lead chromate, the contamination of the adsorbing surface was reduced and the adsorption progressed almost as rapidly as with dyes. It seemed natural to suppose that this greatly increased effect might be at least partly electric in origin, the chromate anion being attracted by the divalent cation. To test this, equivalent concentrations of sodium hydroxide were used instead of lead nitrate. The adsorption was nearly as great as with the lead nitrate. In confirmation, aluminium sulphate was employed as a trivalent cation. If the adsorption is electrical, the effect should be very much greater in this case. This was fully borne out in the result. A narrow perfectly colourless zone immediately became visible below the lead chromate and the adsorption progressed at least as rapidly as with the dye experiments. Fig. 5 illustrates the condition after two days, with $N/100$ dichromate and aluminium sulphate.

The suggestion is indicated that the mordanting effect of aluminium salts is electrical in origin. The results are also interesting in connection with Harrison's [1911] electrical theory of dyeing. It may also be pointed out that Lewis' experiments [1908] which showed that the surface tension of

water-oil films decreased with increase of temperature, do not necessarily apply to adsorption by solids. If the surface tension of the solid-liquid is due to unbalanced molecular attractions at the common boundary, and if the thermal expansion of the liquid is greater than that of the solid, the unbalanced forces will increase with rise of temperature thus augmenting the surface energy and consequently the adsorption. In confirmation it will be noted that in the case of two liquids such as ether and water, one of which has a much greater thermal expansion than the other, the interfacial surface tension has been found to increase with rising temperature [Antonov, 1907]. The influence of temperature on adsorption by solids has not been studied. But Freundlich [1907] found that charcoal adsorbs more acetic acid from water at higher temperatures, and Pelet and Grand [1907] found the same for methylene blue and crystal ponceau adsorbed by silica, kieselguhr and charcoal. It may be noted, however, that, with solids such as fibres having very great specific surface, their thermal expansion would be greater than in a less disperse state owing to the comparatively large volume of the solid in the surface layer where the intrinsic pressure is released.

In all these adsorption experiments the exceedingly narrow zone immediately beneath the adsorbent is always distinctly less coloured than the remainder of the exhausted region. This is more noticeable in the actual specimens, but can be made out in the photographs.

Numerous facts observed during experimental study appear to lead directly to the assumption of adsorption. The following are particularly striking:

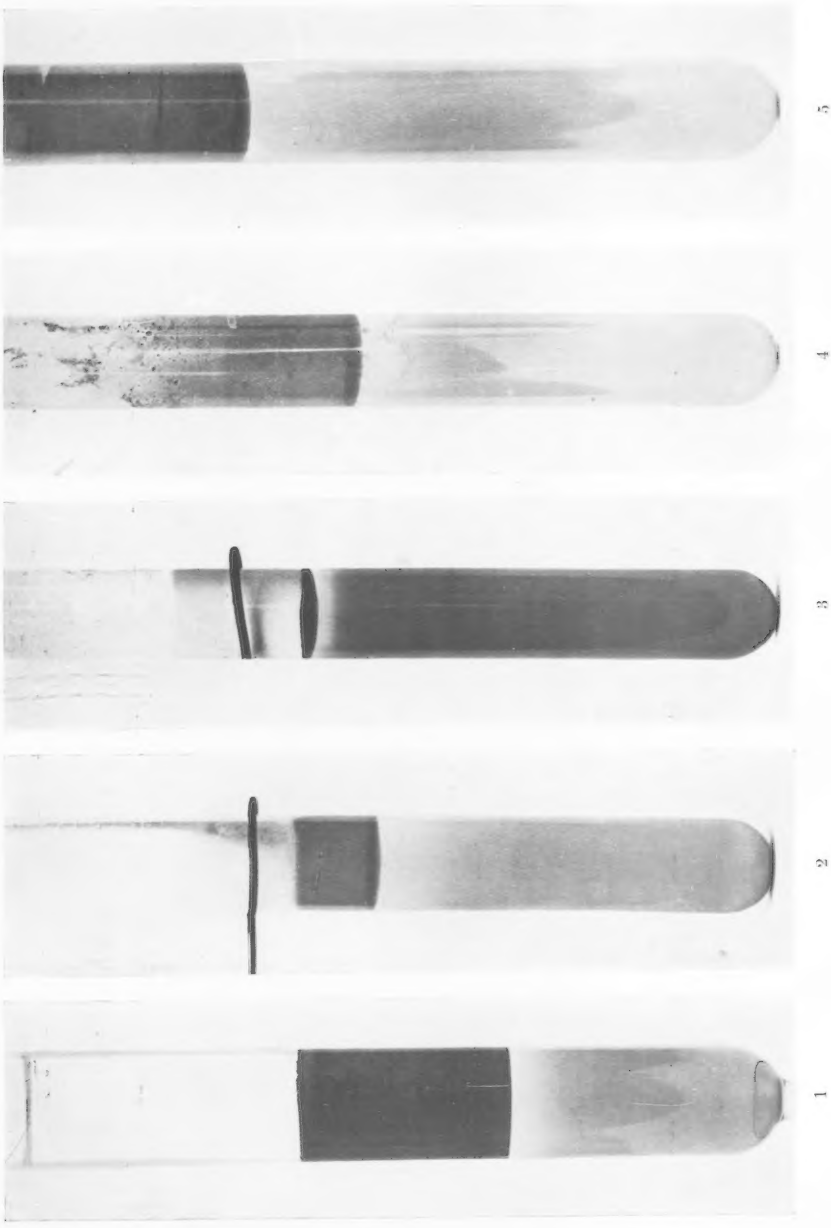
(1) The formation of bands usually ceases long before the bottom of the tube is reached. The hypotonic reagent is invariably so strong that this effect can only be due to the solute in the gel having been exhausted from the lower regions of the tube. This fact also controverts the deduction from the supersaturation theory that the diffusion of the solute in the gel is negligible.

(2) With certain more colloidal precipitates, e.g. manganese sulphide in agar, a tendency is observed to form large spherical aggregates which have much the same structure and density as the bands, except that they frequently show concentric banding. When one of these spherical aggregates begins to form in the zone where a band will shortly appear, the band does not extend to join the sphere, but a spherical cavity, some 2 or 3 mm. wide, is left surrounding the concretion from which the surface of the cavity is everywhere equidistant. This effect must be due to the solute having been exhausted from the neighbourhood of the aggregate. Indeed its spherical form almost certainly implies that nutrient material has accrued equally from all directions.

(3) A similar phenomenon occurs frequently with certain reagents in agar gel. The hypertonic solution accidentally flows down between the gel and the glass, forming a new base from which it diffuses into the gel producing a precipitate with a surface convex inwards, the direction of growth being thus at right angles to that of the precipitate forming down the tube from the upper surface of the gel. It is then observed that this latter precipitate does

not form in the neighbourhood of that growing from the side. It leaves, instead, a clear zone between them which increases in width downwards as the accidental precipitate has had longer time to exhaust the gel in its vicinity.

An interesting feature is sometimes observed in these experiments which appears to afford direct evidence of the energy of the diffusing particles of the solute in the gel. When the agar gel is prepared by straining through muslin, instead of filtering, it has a slightly cloudy, or more opalescent appearance, which is probably due to the presence of larger gel aggregates. During the growth of the first continuous column of e.g. lead chromate in the tube, the region of gel immediately beneath the precipitate loses not only its colour, but also its opalescence, as if the cloudiness had begun to subside, and the clear space gradually extends towards the bottom of the tube. It seems that in such cases the more agglomerated particles of the gel are broken up by the impacts of the rapidly moving solute particles in their diffusion towards the precipitate. In some cases solid particles appear actually to be driven through the gel towards the precipitate. These may be either larger portions of more or less gelatinous matter which have escaped straining, or, apparently they may even consist of particles of the precipitate. It frequently happens with a heavy precipitate that it appears gradually to settle down through the gel. Possibly this may be due to some bacterial or other alteration in the structure or substance of the gel by exposure to the atmosphere. But cases in U-tubes hardly admit of this interpretation. The following is a typical experiment. $N/30$ NaCl and $N/10$ $AgNO_3$ solutions were poured into the respective limbs of a U-tube the lower portion of which was filled with pure 1 per cent. agar gel. With the meeting of the reagents in the gel merely a slight diffuse precipitate was produced at first. After two days this filled the area of the tube for a distance of about 1 cm. and had become somewhat less tenuous. The edge of the precipitate towards the sodium chloride had now developed a distinct, though not very dense, disc of silver chloride. The position of this disc was marked on the tube. The disc then expanded into a column of precipitate which formed continuously from this point, gradually becoming more dense. In the meantime, however, the light diffuse precipitate gradually diminished and in four days had practically disappeared. In thirteen days even the commencement of the denser part of the column had become distinctly less marked and had shifted gradually through about 1·5 mm. At this point the concentration of the silver nitrate would probably have been about $N/50$. The disappearance of the precipitate must either have been due to peptisation by this dilute solution, or to the tiny particles having been driven through the gel by the bombardment of the silver nitrate molecules. In this experiment the denser edge of the growing column attained a maximum and appeared as a distinct disc of dense precipitate at about 12 mm. from the commencement of the column. The less dense column continued to grow with the production of a series of such denser bands. The first five were equally spaced, about 1·5 mm. apart, the sixth, at about twice that distance from the


5
4
3
2
1

previous band, was the last observed thirteen days from the beginning of the experiment. This production of equally spaced discs is worthy of note, since it has been argued that agates with equidistant bands could not have been due to diffusion into gels. Another similar experiment with 5 cc., respectively, of $N/10$ K_2CrO_4 and $N/5$ $Pb(NO_3)_2$ gave eight successive discs with clear spaces between, all approximately 1·15 mm. apart, followed by a further series 0·9 mm. apart.

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IV. ON THE ASSOCIATION OF ANTITOXINS WITH THE PROTEINS OF IMMUNISED HORSE SERUM.

By ANNIE HOMER.

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The data compiled from results obtained in the routine concentration of antitoxic horse sera show that the average loss of antitoxin, due to its association with "salt-insoluble" globulin, is greater for antitetanic than for antidiphtheritic plasma or serum thus:

Method of concentration employed	Percentage loss of antitoxin experienced with	
	Antitetanic plasma	Antidiphtheritic plasma
Banzhaf-Gibson	22	17
Banzhaf (1913)	18·5	12·5
Homer (1916, 1918)	7·5	2·5

In each of the above processes the proteins of the plasma or serum, prior to their fractional precipitation by ammonium sulphate, are subjected to a heat-denaturation which not only increases their precipitability by sulphates but also leads to a conversion of "salt-soluble" protein into a "salt-insoluble" condition.

A comparatively low value for the extent of the denaturation renders the proteins of the euglobulin-pseudoglobulin zone "salt-insoluble"; the progressive increase in the denaturation causes a corresponding conversion of the fractions of the pseudoglobulin precipitated at successively increasing concentrations of ammonium sulphate and, finally, with the further increases in the extent of the denaturation the albumin fractions of the serum proteins are also rendered "salt-insoluble."

From these observations it is evident that, in the concentration of sera, provided there has been no destruction of antitoxin during the heating of the serum, the losses of antitoxin are regulated by the extent to which the heat-denaturation has converted the antitoxin-bearing proteins into a "salt-insoluble" condition.

My previous work has demonstrated that, in the techniques adopted in the above-mentioned routine methods, the heat-denaturation is sufficient to convert the proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. These conclusions, taken in conjunction with the inferences to be drawn from the data recorded above, indicate that the association of anti-

toxin with the unheated proteins of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

In order to furnish further evidence on this point a detailed study has been made of the proportional precipitation of antitoxin and protein by the addition of progressively increasing amounts of sulphate to *antitetanic* and to *antidiphtheritic* plasmas respectively.

It was found that the labour entailed in the investigation was considerably shortened by the substitution of sodium sulphate for ammonium sulphate in the fractionation of the plasma. For, with the use of sodium sulphate in contradistinction to the more toxic ammonium sulphate, there was no necessity for the dialysis of the protein precipitates or of the filtered sulphated plasmas prior to the estimation of their antitoxin content.

The method of procedure was as follows: to separate volumes of unheated antitetanic and antidiphtheritic plasma was added solid anhydrous sodium sulphate in amounts ranging from 9 to 12 per cent., i.e. in amounts sufficient to precipitate the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones. The stoppered bottles containing the sulphated plasmas were heated in a water-bath at 35° until complete solution of the sulphate had been effected. The liquids were then filtered, the respective precipitates being washed on the filters with an aqueous solution of sodium sulphate of the same concentration as that of the sulphated plasma thus filtered.

The antitoxic contents of the filtrates and of the precipitates, the latter being emulsified in a measured volume of water, were estimated in the usual way and, after making the necessary corrections for dilution, etc., data were obtained as regards the percentage precipitation of the respective antitoxins at each stage with the sulphate.

Recent work has shown that the precipitation of the serum proteins and of the antitoxin at a given concentration of ammonium sulphate or of sodium sulphate is somewhat greater from plasma containing cresylic acid than from non-cresylised plasma. I have therefore included in Table I the data obtained from the precipitation of cresylised and of non-cresylised samples of antidiphtheritic and antitetanic plasmas respectively.

Table I. *Showing the percentage precipitation of the antitoxins of antitetanic and of antidiphtheritic horse plasma by anhydrous sodium sulphate.*

Percentage addition of sodium sulphate to the plasma	Percentage precipitation of antitoxin with the protein precipitated by the sulphate from			
	Non-cresylised plasma		Cresylised plasma	
	Antitetanic	Antidiphtheritic	Antitetanic	Antidiphtheritic
9	16	0	16	0
10	16	5	16	4
11	16	5	27	4
12	30	16	35	21

The study of the results given in the table show that both in cresylised and in non-cresylised plasma the precipitation of antitoxin with the proteins

of the euglobulin-pseudoglobulin zone is greater in antitetanic than in antidiphtheritic plasma.

A preliminary study has also been made of the proportional precipitation of the antitoxins of *antidysenteric* and of *antimeningitic* horse plasma with the various protein fractions, and data have been obtained with regard to the association of the respective antitoxins with the "salt-soluble" globulins from the unheated and from the heat-denaturated plasmas.

(a) *The isolation of the "salt-soluble" globulins from the unheated plasmas.*

To a measured volume of plasma was added an equal volume of a saturated solution of ammonium sulphate. The sulphated plasmas were filtered and the precipitates, after having been well washed with a half-saturated solution of ammonium sulphate, drained and well pressed, were emulsified in a small volume of water which was subsequently diluted with a saturated solution of sodium chloride until the volume of the emulsion was approximately twice that of the plasma taken for precipitation. Solid salt was added to the liquid which, after complete saturation with the salt, was allowed to stand at room temperature for 48 hours before filtration. The precipitate, consisting of insoluble protein and salt, was well washed with a saturated solution of salt and to the filtrate and washings was added 0·25 per cent. of glacial acetic acid. The acidified liquid was filtered and the precipitate was pressed and dialysed in the usual way.

(b) *The isolation of the "salt-soluble" globulins from the heat-denaturated plasmas.*

Measured volumes of the plasmas used in (*a*) were adjusted in the usual way so as to ensure, during the heating process, the conversion of the "salt-soluble" proteins of the euglobulin-pseudoglobulin zone into a "salt-insoluble" condition. The adjusted plasmas, contained in stoppered bottles, were heated in a water-bath at 58° for 5 hours. To each of the heated liquids was added an equal volume of a saturated solution of ammonium sulphate and the ensuing precipitate was then subjected to the same treatment as that described above for the corresponding precipitate from the unheated plasmas.

The comparison of the relative antitoxic contents of the original antidysenteric plasma and of the "salt-soluble" globulin fractions isolated therefrom, was made in terms of a laboratory standard toxin prepared by drying Shiga bacilli *in vacuo*. The determinations of the antitoxic values of the original antimeningitic plasma and of the "salt-soluble" globulins were carried out by the method advocated by Hitchens and Robinson [1916].

The values thus obtained for the titre of the respective liquids cannot be relied upon to the same extent as those furnished by the generally accepted methods for the testing of antitetanic and of antidiphtheritic sera. For this reason the data furnished in Table II are open to criticism. However, that a certain amount of reliance can be placed in these results is shown by the fact

that, as was anticipated from our previous knowledge of the heat-denaturation of antitoxic sera, the extent of the association of antitoxin with the "salt-soluble" globulins is considerably less in the heat-denaturated than in the unheated plasmas.

Table II. *Showing the percentage of the total antitoxins precipitated with the "salt-soluble" globulins of antidysenteric and antimeningitic horse sera.*

Plasma	Percentage of the total antitoxin associated with the "salt-soluble" globulins of the	
	Unheated plasmas	Heat-denaturated plasmas
Antidysenteric	50	30
Antimeningitic	30	10

The results depicted in the table are therefore sufficiently striking to lead us to the conclusion that, contrary to our experience with antitetanic and antidiphtheritic plasma, the bulk of the antitoxins in antidysenteric and antimeningitic plasma is associated with the proteins of the euglobulin and of the euglobulin-pseudoglobulin zones.

Attempts to furnish data in regard to the association of the antitoxins of antidysenteric and of antimeningitic sera with the proteins precipitated at successively increasing concentrations of sodium sulphate have been postponed until more exact methods of testing the titre of these sera are forthcoming.

The results of the present investigation are of interest as they show that the various antitoxins are adsorbed by different protein fractions of horse serum, a phenomenon which can be regarded as an indication of differences in the molecular composition of the respective antitoxins.

REFERENCE.

Hitchens and Robinson (1916). *J. Immunol.*, **1**, 345.

V. THE GUANIDINE CONTENT OF FAECES IN IDIOPATHIC TETANY.

By JOHN SMITH SHARPE.

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(*Received December 22nd, 1919.*)

Since the condition of tetany has sometimes been ascribed to gastro-intestinal disturbances, and since the work done in this Laboratory has associated it with guanidine intoxication [Noël Paton and Findlay, 1917], the possibility that it may be due to absorption of guanidine from the intestinal tract has to be considered. Against this possibility is the fact observed by Findlay that large doses of guanidine may be administered by the mouth to kittens without producing symptoms. On the other hand it is possible that guanidine may be partly excreted from the intestine. The work of Burns indicates that it is not all eliminated in the urine [Burns, 1917].

I have therefore made some examinations of the faeces of normal children and of those suffering from tetany. The material was procured from The Royal Hospital for Sick Children, and I am indebted to Dr Leonard Findlay.

Method.

Entire specimens of daily excretion of faeces were collected from various normal and tetany cases and weighed. Aliquot parts were subjected to the following process for the extraction of bases, within an hour of being passed, to avoid any possible decomposition.

30 to 50 g. of the moist faeces were mixed thoroughly in a mortar with 100 cc. of water. A little chloroform and thymol were added and the whole placed in a dialyser with a parchment membrane. This was surrounded by 200 cc. of water, put in a cool place and dialysis continued for three days. The dialysate was then washed into a beaker, more water added to the first beaker and dialysis continued for a further two days. The two dialysates were united and evaporated down to a syrup. Ammonium, potassium and excess of sodium were taken out with chloroplatinic acid in the usual way, and the platinum removed by sulphuretted hydrogen. The sulphuretted hydrogen was then evaporated off and the bases precipitated in the filtrate with saturated alcoholic picric acid.

Almost immediate formation of crystals was observed. Small bunches of yellow needle-shaped crystals formed. These were very insoluble in water and gave long needle-shaped forms on recrystallisation from water.

The melting point of these crystals was 230° and the nitrogen content was 25 per cent. These figures agree closely with those for dimethylguanidine.

A loss of about 5 per cent. is incurred by this method, but by using the quantities above-mentioned this is constant and may be corrected for. This loss was determined by adding a weighed quantity of a guanidine salt to normal faeces and proceeding as above. The differences indicated in the tables below are far outside any loss in analysis. The process has the advantage in this case of easily eliminating all the suspended matter without the use of precipitants, which is very troublesome in faeces.

Tetany faeces. Age, from 1–2 years.

No.	Daily wt. of fresh faeces	Dimethylguanidine per cent.	Daily excretion of dimethylguanidine
1	25 g.	0·080	0·020 g.
2	30 „	0·074	0·022 „
3	22 „	0·070	0·015 „
4	20 „	0·078	0·016 „

Normal faeces. Age, from 1–2 years.

1	15 „	traces	—
2	12 „	0·028	0·003 „
3	13 „	0·018	0·002 „
4	16 „	minute traces	—
5	18 „	„ „	—
6	11 „	„ „	—

Conclusions.

The average percentage of this base in the four tetany cases examined was 0·075 per cent. of the moist faeces. In the six normals only faint traces were detected, averaging 0·007 per cent.

The average daily excretion of guanidine by the bowel in children suffering from tetany according to the present investigation is 0·018 g. as dimethylguanidine.

The work was done under The Medical Research Committee.

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Noël Paton and Findlay (1917). *Quart. J. Exp. Physiol.*, **10**, 3 and 4, pt. 4.

VI. ON THE COLOURS OF TWO SEA ANEMONES, *ACTINIA EQUINA* AND *ANEMONIA SULCATA*.

PART I. ENVIRONMENTAL.

By RICHARD ELMHIRST.

PART II. CHEMICAL.

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(*Received December 24th, 1919.*)

(With Plates III, IV.)

Many and varied are the theories that hold the field of colour phenomena in nature. This is to be expected, for although a large amount of work has been published on this subject, very little of it takes one further than the surface. There are the theories of Darwin, Poulton, Wallace, Eimer, Simroth, etc., which are all based on a hypothesis of structure in relation to natural selection or environment, the works being so well known that it is unnecessary for us to enter into any detail here. The aim of the present investigation is to endeavour to obtain an interpretation for certain colours.

Part I.

Environmental.

The commonest British Actinian is *Actinia equina* L., occurring on rocks and stones all around our coasts. Several colour varieties have been described and recorded in varying abundance from many localities. The species is abundant in the Clyde area: the prevailing form being the normal liver-red type, with bright blue "marginal spherules" and an equally blue border round the base, var. *hepatica* [Gosse, 1860, Pl. 6, fig. 2].

Ideal Habitat.

The ideal habitat for this species, as shown by the accompanying table, is about half-tide mark among the dense growths of *Ascophyllum* and *Fucus serratus*. The luxuriant growth of these weeds provides shelter and moisture during ebb and shade and protection from the force of the waves during flood. The presence of these weeds is, however, not essential because *Actinia* may occur abundantly on shelving rocks where they are replaced by *Chondrus* and *Gigartina*, short crisp weeds some three to four inches high.

In rock pools *Actinia* may occur at any tidal level up to and rarely above high water mark.

Vertical distribution of *Actinia*.

Sea-weed zone	Exposed upper surface of stones	Sheltered
Pelvetia canaliculata	—	Few
Fucus platycarpus	Rare	Few
Fucus vesiculosus	Few	Frequent
Ascophyllum nodosum	Common	Common
Fucus serratus	Common	Common
Laminaria digitata	Few	

Colour.

Within the limits of var. *hepatica* there are two shades of colour. (1) A deep red, occasionally tending almost to blackness, which occurs in exposed situations and (2) a paler bright red which occurs in shelter under sea-weed, below stones and in crevices. This agrees with Walton's observations on *Actinia equina* at Aberystwyth and in South Devon [1911, pp. 229 and 239]. Farquhar's on *Actinia tenebrosa* in New Zealand [1898] and Saville-Kent's on the coral *Euphyllia* [1893]. Of the other colour varieties described by Gosse, the brown var. *umbrina* forms less than 1 per cent. of all examined except in a few favoured localities where a pale var. *umbrina* seems to have become established. These varieties (*hepatica* and *umbrina*) merge through red browns so that it is often a matter of considerable difficulty to decide how to describe a particular specimen. Red browns and browns both vary in depth of colour according to the exposure of their surroundings. Two other varieties, the sea-weed coloured *olivacea* and red with green marks *fragacea*, are rare—less than 1 in 5000. Clyde var. *fragacea* are always small, 1 to 1·5 cm., apparently never attaining the inches of the Devon and Cornish specimens.

Relationship of Colours.

It is well known that the distinct colour varieties are connected by intermediate shades, which suggests that the pigments are chemically related to each other and probably derivatives from the same source. This is also borne out by several experimental observations of individuals changing colour, notably the fading of brilliant markings. Similar changes have been recorded for *Madrepora prostrata* [Saville-Kent, 1893].

In 1910 a normal *A. equina* was placed in a well-lit glass-sided aquarium. By the next year this specimen had passed from red through red-brown, brown-green to a deep olivaceous-green, i.e., had assumed the coloration of var. *olivacea* and attained a diameter of from 4 to 6 cm. This assumed coloration has persisted through a number of generations from 1911 to 1919. The young are usually pale greyish green about the shade of Gosse's var. *glauca*. A few, however, show a faint reddish tinge but in all cases the final colour has been that of var. *olivacea*.

Experiments are now in progress (1) to repeat the assumption of the green colour and (2) to reverse the process and get the var. *olivacea* to reassume the red of var. *hepatica*. With the former aim, three normal red var. *hepatica* were placed on May 10th under surroundings precisely similar to those in which the change *hepatica* to *olivacea* took place in 1910–11. Now, after five months, they have lost much of their original red and the tentacles are completely brown which, so far, is a repetition, though slower, of the 1910–11 process. With the latter aim a number of var. *olivacea* are being subjected to alternate dryness and submersion, while a second set are being kept in an artificial rock pool cut in the local sandstone. At present, after three months, no very definite results are noticeable but a suggestion of redness is appearing above the blue basal border in two cases.

Inheritance of Colour.

It is interesting to note that this assumed coloration has so far bred true. Gosse mentions [1860, pp. 178–9] that vars. *cerasum* and *chiococca* breed true. The same is suggested by the frequent occurrence on the shore of the young of any particular variety near an adult of that variety. At times a variety may establish itself in a suitable locality.

There is a rock-sheltered corner on the east shore of Cumbrae at the *Fucus serratus* level where *Metridium dianthus* was always found, both the red and white varieties (1906–15). On visiting this place in 1919 *Metridium* was found to be absent and in its place were a large number of pale brown *A. equina*, in colour between Gosse's vars. *umbrina* and *ochracea*. There were some 250 specimens of this variety which had established itself for a number of yards to the almost total exclusion of the normal red.

Colour Significance.

The significance of these colour forms has been ably discussed by Walton [1911] who classifies the coloration of actinians as

(1) Warning;
(2) Aggressive;
(3) Protective;
(4) Colours with some special physiological significance.

In *Actinia equina* the brilliant blue of the "marginal spherules" has been generally accepted as warning coloration. This may well be so, but unfortunately we have no observations bearing on the matter, although the fact that the normal colour often contrasts strongly with the environment may bear this interpretation. Because of this environmental colour contrast it is improbable that the aggressive coloration suggestion holds true although crustacea in aquaria have occasionally been seen to stumble onto an *A. equina*. That the normal colour is protective to any extent will be disproved by a single walk along the shore, despite the redness of the sandstone in several

localities. If the coloration were protective varieties should occur in definite association with environment, e.g. rock pools filled with *Cladophora*, backgrounds of sandstone, basalt or limestone, yet the normal red prevails in all these surroundings. In the *Cladophora* pools, particularly, dark reds which presented a marked contrast to their surroundings have been found occurring abundantly, 800 specimens without a single variety present. This brings us to colours with some special physiological significance, which interpretation is strongly suggested by (1) the variation in colour correlated with exposure already mentioned, and (2) the well-known fact that brightly coloured specimens when kept in aquaria and therefore continually submerged tend to lose their distinctive coloration. The obvious inference seems to be that the pigment is a light screen which has some physiological significance, possibly respiratory, since change of colour often takes place when specimens are kept continually submerged and not exposed to the alternate ebb and flow of the tide.

Anemonia sulcata (Pennant)=*Anthea cereus* (Ellis and Sol), occurs abundantly in suitable localities all round our southern and western coasts. In the Clyde, the normal "umber brown" is frequent on *Laminaria*, where it closely resembles the background of sea-weed: a case of aggressive coloration which obtains "much food in the form of deluded crustacea."

The ideal habitat of this species is a bed of *Laminaria digitata* sheltered from heavy waves and having a good exposure to sunlight. The first condition is favourable to the species because it is incapable of strong attachment and the second is essential for the life of the symbiotic algae inhabiting the tentacles. Excellent examples of this ideal habitat are seen in Castle Bay, Little Cumbrae, and between the Eilans in Millport Bay; the species being abundant in both places.

The need of direct strong sunlight is very well illustrated on the walls of the cambers in Gibraltar harbour, where on the walls running east and west, the north sides are practically devoid of *Anemonia* whereas the south sides are covered with thousands of fine specimens of the beautiful rose-tinted variety *smaragdina*.

Summary.

1. The ideal habitat is at half-tide level.

2. Intensity of colour varies with exposure to light.

3. Individuals may show (environmental) colour change.

4. Individuals breed true.

5. A given variety may establish itself in suitable environment. In the present absence of evidence of the actual difference in environments it seems that one shade of colour may be as effective for the success of the species as another.

6. The coloration seems to have a special physiological function as a light screen.

Part II.

Chemical.

Apparatus used.

A Browning microspectroscope, with an inch objective and condenser, was used. The solutions of the colours were placed in small flat-bottomed glass tubes of about 10 cc. capacity. A compressorium was used for the examination of the fresh tissue which was cleared in glycerol. The positions of the various absorption bands and screens were determined by the Fraunhofer lines on a comparative solar spectrum.

Actinia equina.

1. *The ether-alcohol-soluble portion.* In the first part of the chemical work the interest was centred round substances of the nature of lipochromes. The fat solvents were all tried with varying success although the extracts were weak. In all cases the best extract of the ectodermic lining where the colouring matter was contained, was obtained by treatment with a mixture of three parts ether to one of alcohol. This gave in every case strong extractions of the red, brown-red or brown-yellow colours. On evaporation, oily drops were left corresponding in colour with the original coloured solution. These did not mix with water but lay on the surface as an oily layer. No haemoglobin derivatives could be demonstrated in this colouring matter; it is therefore not respiratory in function, at least as an oxygen carrier. Acids produce no change in the colour but on the addition of alkalies, NaOH for example, a brown colour resulted and the spectrum was changed. (See Plate III, A, spectrum 6.) This is important as it involves the possibility of acid or alkali playing a part in the colours of these animals, but whether this is produced by food or light is a question to be considered. There is one point that is significant in the absorption spectra of Plate III, A, that is the screening over the green and red portions of the spectrum.

According to Grotthus' law (1819) photo-chemical action cannot take place unless light is absorbed. Now Engelman has shown that red sea-weeds show the greatest carbon assimilation in the green and by the spectrum they show the greatest absorption. The chlorophyll in these red weeds is modified and acts as an optical sensitiser. This same effect is produced by light of various wave lengths, provided they are absorbed.

Again photo-chemical action seems not to depend on molecular construction, for with colouring matters ranging from red, yellow to blue, the molecules of which are not arranged in the same way, it has been shown that in every case where light is absorbed iodine can be set free by the released oxygen from potassium iodide, independent of absorption wave-length or position on the spectrum [Wager, 1914].

It will readily be seen that there is in these anemones a substance absorbing light in the green and red parts of the spectrum and generally resembling

chlorophyll in its action on light. This substance although not showing the distinctive bands of chlorophyll gives absorption wave-lengths in almost the same position as the maximum absorptions of chlorophyll.

Furthermore it was proved experimentally by Wager's method [1914] that these red and brown substances produce photo-chemical action with the release of active oxygen.

By the use of the fluoroscope a faint fluorescence is seen in the red almost over the lines *B, C*. This is in the position of maximal energy of the chlorophyll system as found by Engelmann and Timiriazev. According to Kerner red fluorescent substances have the power to transform the blue rays to red and ultra-red rays or in other words, rays of light into chemical rays. It is highly probable therefore, that such an action takes place in these anemones and as such may be concerned chiefly in their constructive metabolism.

This has also a bearing on the respiratory pigment which in these animals is very small in amount and must act more as a fixer than a carrier of oxygen.

Thus it will be seen that adequate conservation of the light is all important and that the colouring matter by virtue of the heavy screen towards the blue end affords protection against the harmful ultra-violet light.

In brown specimens a powerful screening of the blue and violet end is again secured by a brown substance soluble in ether-alcohol of the nature of a lipochrome. In spectrum 4, Plate III, A, it will be seen that even with a thin layer the screening towards the violet is very marked. The other parts of the screen behave in very much the same way as those of the red type.

In the lighter sheltered red and brown anemones there is only a variation in the intensity of colour but the photo-chemical nature remains the same.

2. *The respiratory pigment.* Portions of the ectoderm of a red and a brown anemone were cleared in glycerol and examined spectroscopically in a compress. Two bands were visible as indicated in Plate III, B, spectra 1 and 3. It will be noticed that in spectrum 3 the bands are a little further towards the blue end. MacMunn [1914] found a somewhat similar displacement in his brown specimens but he noted a band to the left of the *D* line. This could not be found with sufficient certainty to map. The presence of this band may vary with the state of oxidation of the respiratory pigment, but all our specimens were taken from fresh sea-water and used without delay. The demonstration of reduced alkaline haematin and also of haematoporphyrin is positive, Plate III, B, spectra 2, 4, 5, and in this we confirm MacMunn's findings.

The tests have to be carried out with great care as there is present an extremely small quantity of respiratory pigment. This was further proved by testing the oxygen content of the tissues, which gave spectroscopic evidence of haemoglobin derivatives, by the Barcroft [1914] method. Only 0·25 per cent. of oxygen by volume was given off by the ferricyanide from 5 g. fresh tissue as compared with mammalian blood which gives an average of from 17 to 18 per cent. evolved oxygen.

Experiment with a young red Anemone. A young red anemone was placed in fresh sea-water and screened from white light by various coloured glasses. When screened by either red or blue it moved into the yellow or any white light portion within a few hours. This experiment was repeated many times, the animal never failing to seek yellow or white light as rapidly as movement in these animals is possible. When the anemone was left altogether in white light it would remain in the same position for days.

The following are the absorptions of light by the coloured glasses used in this experiment:

Red. Nothing in the red, a strong dark band in the yellow and a heavy screen from the green to the end of the visible spectrum.

Yellow. A faint shading in the blue end.

Blue. A strong screen over the red and orange, a fainter screen from the mid-yellow to the end of the green and nothing in the blue.

Thus with these red and blue glasses the very light that the coloured ectoderm absorbs has been withheld from the anemone, making it necessary for the animal to change its position in order to obtain the rays of light most useful to it.

Anemonia sulcata.

1. *Ether-alcohol-soluble portion of ectoderm* (free from algae). A faint yellow colour was obtained giving no marked absorption spectrum, only a faint shading in the violet region.

2. *The respiratory pigment.* Alcoholic extracts of the tentacles and mesenteries of this anemone gave a greenish-yellow liquid which showed very characteristic absorption bands. The ether extract gave almost identical bands, the only difference being a change in the intensity of the bands on either side of the *C* line. In the alcohol solution the band to the left of the *C* line, as shown in Plate IV, C, spectrum 1, is very intense. Spectra 2 and 4 (Plate IV, C), the layers of which are only $\frac{1}{20}$ the thickness of those of 1 and 3, still show this band very markedly. The positions of the bands in ether solution show a very close resemblance to those of β-chlorophyll of the green leaf. The bands on either side of the *C* line occupy the position of the maximum intensity of the chlorophyll system, while in the thin layer the band between *F* and *G* is in the position of the more refrangible band of carotin or xanthophyll. We confirm MacMunn in these findings and also that this chlorophyll-like substance comes from the symbiotic algae contained in the tentacles and mesenteries of this animal. (See Microphotograph, Plate IV, D.)

Further no haemoglobin derivatives could be demonstrated by us in the tissues and it seems conclusive that this chlorophyll is here as an optical sensitiser to supply active oxygen for the use of the anemone. An experiment which may support this is as follows. We screened an anemone from light in a glass vessel containing sea-water. It climbed up the side until it could obtain the maximum daylight available. Whether this is due to a vital

necessity of the anemone or to a stimulus produced by the heliotropic action of the algae on their own behalf are questions which would be difficult to answer. Again Geddes [1882] has shown that *Anemonia sulcata* when placed in bright sunlight evolved oxygen in quantity sufficient for determination by gas analysis apparatus.

Previous Work.

Moseley [1887] during the "Challenger" expedition made a large series of spectroscopic examinations of the various invertebrate colours. From our present standpoint his work upon the pigments of the Coelenterata whether respiratory or otherwise is of more direct importance. He isolated from certain species of Actiniae a madder red colour. In fresh condition this colour yielded a well defined absorption spectrum. Three bands were visible, one in the green and the others toward the less refrangible end. For this pigment the name polyperthyrin was proposed. MacMunn [1914] found that this polyperthyrin was to all appearances identical with haematoporphyrin although he indicates there is only spectroscopic evidence for this similarity. Moseley's polyperthyrin is very stable even on exposure to light. It is soluble in moderately strong hydrochloric and sulphuric acids. It is insoluble in water, glycerol, alcohol and ether. The colour in strong concentration of the above acids gave a screen from the *D* line extending all the way towards the violet end. When this solution was further weakened two distinct bands appeared close to and on opposite sides of the *D* line. The shading towards the less refrangible end was also lessened. This colouring matter was precipitated on the addition of alkalies as a burnt sienna flocculent deposit which produced on redissolving in acids the three-banded spectrum of the fresh substance.

According to Moseley [1887] polyperthyrin has been found in eleven different species comprising seven genera of Coelenterata. McKendrick [1881] examined the pigments of *Cyanea* from aqueous infusions of the animal. Two bands were observed, one in the orange and the other in the red. This pigment has been termed cyanein.

In Griffiths' *Physiology of the Invertebrata* it is stated: "There is no doubt that the chief mode of respiration of Coelenterates is by means of the ectodermic lining."

MacMunn [1885] devoted much time to the examination of respiratory pigments in Actiniae. The solid portions of *A. equina* were first examined in a compressorium by the microspectroscope. A band was seen which was not unlike that of reduced haemoglobin, and two other bands were noted nearer the violet end. In brown specimens he noted that the prominent band was nearer the violet and also that in some there was a band close to the left side of the *D* line. This pigment has been named by MacMunn actiniohaematin. Actiniohaematin is insoluble in the fat solvents but dissolves in glycerol. It is extracted by treatment with alcoholic potash solution with a change in condition. The spectrum thus seen was very like that of alkaline haematin

which gives a band at *D*. When reduced with ammonium sulphide two bands were observed, being to all appearances the spectrum of haemochromogen or reduced alkaline haematin.

Again the spectrum of haematoporphyrin was obtained by digesting the ectoderm of an *Actinia* in sulphuric acid and filtering through asbestos. This was further proved by the addition of ammonia in excess forming the four-banded spectrum characteristic of alkaline haematoporphyrin.

Moseley [1873] isolated from a *Tealia crassicornis* a substance which he called actiniochrome. MacMunn has proved that Moseley's actiniochrome is not the same as actiniohaematin for the products of decomposition are entirely different; actiniochrome could not be converted to reduced alkaline haematin whereas actiniohaematin very easily could. MacMunn concludes therefore that actiniohaematin is respiratory in function and actiniochrome is ornamental.

Again MacMunn has found certain "yellow cells" (symbiotic algae) in the tentacles and mesenteries of *Bunodes ballii* which give the spectrum of a chlorophyll-like substance.

Actiniochrome was found in this species but haemochromogen could not be demonstrated. He remarks that: "The replacement of actiniohaematin by the colouring matter of these 'yellow cells' is of great interest for it appears that they replace the red pigment of other species."

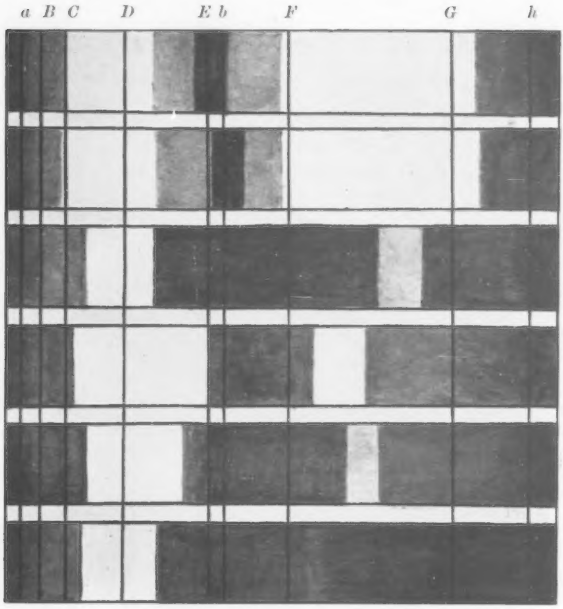
In the genus *Sagartia* MacMunn found actiniohaematin in many species, also the chlorophyll-like substance (chlorofucin) in *Sagartia bellis*. The "yellow cells" which lie in the endodermal lining of the tentacles were responsible for this spectrum. Further he finds that these "yellow cells" suppressed the respiratory pigment. In *Anemonia* "yellow cells" were also found which yielded the chlorophyll-like substance.

Geddes [1882] proposes the generic name of "philozoon" for the symbiotic algae or "yellow cells." In the same paper he gives a detailed account of chlorophyll-containing animals chiefly Coelenterates. He shows that there is a less amount of oxygen evolved by imprisoned algae, i.e. the symbiotic species, than by free living individuals. He concludes that the avidity for oxygen of the animal protoplasm accounts for this difference.

Conclusions.

1. *Actinia equina*. In view of the small quantity of respiratory pigment along with the alcohol-ether-soluble substances, it is concluded that the red and brown colours of these anemones are not ornamental as MacMunn and others hold, but that they act as optical sensitisers producing active oxygen for the use of the animal through the medium of the respiratory pigment. By virtue of the red screen in the position of the maximum absorption of chlorophyll they act as chemical sensitisers. Further there is reason to believe that these animals can make use of the blue and probably the violet rays.

2. *Anemonia sulcata*. In *Anemonia* there is no haemoglobin derivative present, but the animal contains minute algae lining the inside of the tentacles



Actinia from sheltered position, thin layer.

Actinia from exposed position, thin layer.

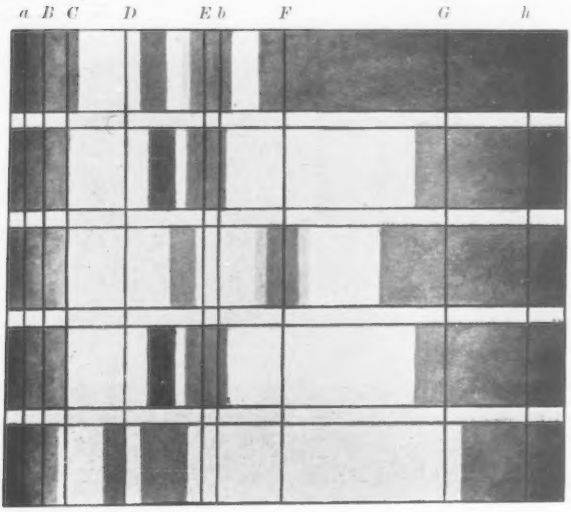
Actinia from exposed position, thick layer.

Actinia, natural brown variety, thin layer.

Actinia, natural brown variety, thick layer.

Solution from 2 treated with sodium hydroxide.

A. *Actinia equina*.
Absorption spectra of ether-alcohol soluble extract of ectoderm.



Ectoderm of dark red *Actinia* in glycerol.

Reduced alkaline solution from dark red *Actinia* showing the haemochromogen spectrum.

Ectoderm of brown *Actinia* in glycerol.

Reduced alkaline solution from brown *Actinia* showing the haemochromogen spectrum.

Ectoderm of red *Actinia* in H_2SO_4 showing the spectrum of acid haematoporphyrin.

B. *Actinia equina*.
Spectra of respiratory pigment.

and mesenteries, which contain a chlorophyll-like substance. By its close similarity to the chlorophyll of the green leaf, the action of light on this body seems to be much the same, namely of optical and chemical sensitisation. Active oxygen therefore would be released by the contained algae and used by the animal if need be.

We have to thank Professor D. Noël Paton for help and advice. The expenses were defrayed by the Medical Research Committee.

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VII. THE PENETRATION OF ELECTROLYTES INTO GELS.

I. THE PENETRATION OF SODIUM CHLORIDE INTO GELS OF AGAR-AGAR CONTAINING SILVER NITRATE.

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Introduction.

"The manifestly colloidal nature of living protoplasm demonstrated *ad oculos* the significance of studies on colloids for Biology," says a distinguished continental plant physiologist [Czapek, 1911], and indeed the importance of investigations on sols and gels from the point of view of physiology needs no emphasis at the present time. An enquiry into the penetration of salts into gels may help towards the solution of problems of permeability in living and dead plant and animal tissues, and it is from this point of view that the experiments described in this and following papers were made.

A number of investigations on the penetration of electrolytes into gels have been made since the time of Graham. As well as the researches of Graham himself may be mentioned those of de Vries [1884], Coleman [1887, 1888], Chabry [1888], Voigtländer [1889], Durrant [1906], Bechhold and Ziegler [1906], Öholm [1913], Vanzetti [1914], and von Fürth and Bubanović [1918].

In the majority of the cases cited above the method employed to study the diffusion of substances into gels consisted in analysing different layers of the gel after penetration of salt had proceeded for a certain time. Working in this way Graham concluded that diffusion of crystalloids proceeds as rapidly in a gel as in water. Voigtländer investigated the diffusion of a number of chlorides in agar gels of different concentrations and came to the conclusion that the rate of diffusion was independent of the concentration of the gel. Öholm investigated the diffusion of potassium chloride from a solution of normal concentration into 2, 5 and 10 per cent. gelatin, the amount of potassium chloride being determined in four successive layers of the gelatin by titration with silver nitrate. Öholm found a progressive decrease in the rate of diffusion with increasing concentration of the gelatin and concludes that as a first approximation the presence of gelatinous substances affects the

diffusion of an electrolyte in the same way as a non-electrolyte in general, such as sugar or glycerol [cf. Öholm, 1912].

The method used by Coleman, Chabry, Durrant, Vanzetti and von Fürth and Bubanović is similar to that introduced by Lodge for comparing the mobilities of ions. The characteristic of this method is that the diffusion into the gel is followed by means of an indicator in the gel. Thus the penetration of an acid may be followed by means of litmus or other indicator; the diffusion of chlorides by means of silver nitrate. The chief researches by means of this method are those of von Fürth and Bubanović [1918], who have followed the penetration of a large number of salts, acids and alkalies into gels of 5 per cent. gelatin and principally 2 per cent. agar-agar. From their experimental results they draw a number of conclusions in regard to diffusion in gels and water and the various factors determining the rate of diffusion.

While von Fürth and Bubanović are quite right in emphasising the importance of these researches for physiology and physical chemistry, it would appear that they have not realised the complexity of the system with which they deal. As an example of this may be quoted their experiment 7, in which the diffusion of hydrochloric, sulphuric and oxalic acids, sodium chloride, and ammonium, potassium, magnesium and zinc sulphates was followed in gels of 2 per cent. agar containing silver nitrate and barium nitrate. From the rate at which the zone of precipitated silver chloride or barium sulphate moves onward conclusions are drawn as to the abnormal behaviour of sulphates compared with chlorides in regard to the diffusion of these substances in gelatin. Now this can only be the case if the silver and barium salts in the gel are without influence on the rate of diffusion of the salts through gelatin, as the concentration of the indicators in the gel is not mentioned and is evidently regarded as having no influence on the rate of diffusion. But from first principles it is not to be expected that this is the case, and the first step in obtaining data as to penetration into gels by the indicator method is obviously to determine how variation of the different factors in the case of one system influences the experimental results.

In this paper therefore the system consisting of an agar gel which is penetrated by sodium chloride is investigated, the various factors, concentration of sodium chloride in the external solution, concentration of gel and concentration of silver nitrate in the gel being varied and the effect of variation of these factors on the penetration of the chloride determined.

Method.

The gel was prepared by dissolving ordinary shred agar in distilled water so as to make a solution $\frac{4}{3}$ times as concentrated as required. A solution of silver nitrate four times as strong as that required in the gel was then added to the warm agar solution the proportions by volume of agar and silver nitrate solution being 3 to 1. The mixture after well stirring was poured into test-tubes to a constant height (generally 6 or 7 cm.) and after the gel had

cooled and set a definite height of the sodium chloride solution was added (this was generally 6 or 7 cm.). The tubes were kept in racks in a dark cupboard in which the temperature was measured by means of a self-recorder. The temperature never varied more than 2° or 3° throughout an experiment.

As the chloride penetrates the gel the silver is precipitated as chloride, the limit of which is sharply marked from the gel below containing no precipitated silver chloride. The distance between the upper surface of the gel and this sharp line of demarcation is called by Voigtländer and by von Fürth and Bubanović the "Diffusionsweg": it will be called here the "penetration," and the movement forward of the silver chloride the "march of diffusion." The penetration of course increases with time, though as we shall see, not at a constant rate. The line of demarcation represents of course a layer of a definite concentration of chloride, namely the saturation concentration of silver chloride in the gel. The saturation concentration of silver chloride in water at 20° is 0·00015 g. of salt in 100 g. of water. It is thus the movement forward of a concentration of sodium chloride of this order of magnitude that is followed by the indicator method.

Experimental Results.

A. *Influence of concentration of sodium chloride.*

In a number of experiments with 2 per cent. agar gels containing some silver nitrate von Fürth and Bubanović have shown that the march of diffusion is more rapid the stronger the solution of salt external to the gel, provided the comparison is made with the same gel.

A large number of experiments have been made in the present research with gels of 0·5, 1, 2, 3 and 4 per cent. agar containing varying quantities of silver nitrate, and the observation of von Fürth and Bubanović as to the influence of concentration in affecting the march of diffusion has been confirmed.

From 30 series of experiments in which the concentration of sodium chloride was the only factor varied three examples will suffice to indicate the influence of concentration of the external salt. The results are shown in the following tables. One series of results is shown graphically in Fig. 1. The others are similar.

Table I. 0·5 per cent. agar gel containing 0·005*N* $AgNO_3$. Each number is the mean of two determinations.

1·2*N* NaCl		*N* NaCl		0·5*N* NaCl		0·2*N* NaCl		0·1*N* NaCl		0·05*N* NaCl	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2·92	13·8	2·92	13·5	2·90	12·4	2·90	10·6	2·88	9·1	2·87	7·9
20·33	38	20·33	37·2	20·32	34·1	20·32	30	20·30	26·2	20·38	22
27·58	44·5	27·58	43·8	27·57	40	27·57	35	27·55	31	27·53	25·8
44·25	56	44·25	54·7	44·23	50·2	44·23	44·1	44·22	38·6	44·2	32·2
70·0	>70	70·0	>70	70·0	64·5	70·0	55·1	70·0	48·1	70·0	40·1

Table II. 2 per cent. agar containing 0·1*N* $AgNO_3$.

5*N* NaCl		2*N* NaCl		*N* NaCl		0·5*N* NaCl		0·2*N* NaCl		0·1*N* NaCl	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2·68	10·5	2·55	7·9	2·42	6·3	2·32	4·8	2·18	2·4	2·10	1·0
4·97	14·5	4·83	11·2	4·70	9·1	4·60	7·4	4·47	4·1	4·40	1·0
23·78	39·2	23·65	25·8	23·5	21·3	23·4	17·1	23·3	10·2	23·2	5·0
29·40	45	29·27	29	29·1	24·5	29·0	19·4	29·9	12·1	29·8	5·5
48·42	58·5	48·28	37·5	48·1	31·5	48·0	25	48·9	15	48·8	7
		71·33	45·5	71·2	38·2	71·0	30·5	70·9	18	70·9	8
		95·4	53·5	95·3	44·5	95·2	35	95·1	25	95	8·2

Table III. 4 per cent. agar containing 0·05*N* $AgNO_3$.

5*N* NaCl		2*N* NaCl		*N* NaCl		0·5*N* NaCl		0·2*N* NaCl		0·1*N* NaCl	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
3·10	13·5	2·98	10	2·83	8·2	2·72	6·5	2·60	2	2·50	1·3
5·27	19·0	5·15	13·2	5·00	11·4	4·88	8·8	4·77	7	4·67	2·5
24·1	39	24·0	29·2	23·8	25	23·7	21	23·6	15·6	23·5	8·5
29·8	43	29·7	33	29·6	28·5	29·4	17·5	29·3	17·5	29·3	10
48·7	52·6	48·6	42·2	48·5	37	48·3	30	48·2	22·2	48·1	13
71·8	65	71·1	52	71·6	45	71·3	37	71·3	27	30·5	16·2
		95·6	64	95·5	53	95·4	42·5	95·2	30·5	95·2	18·4

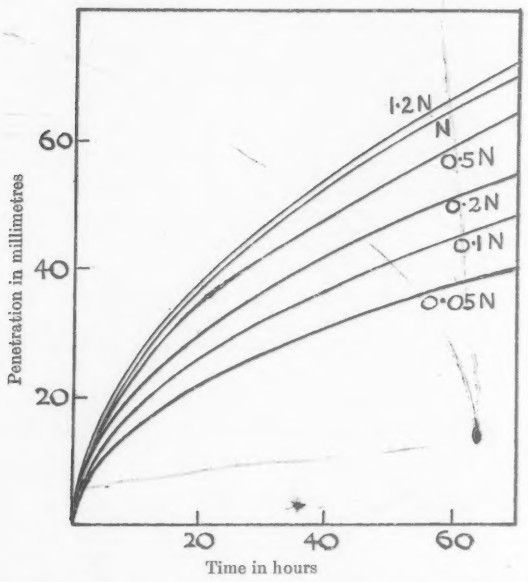

Fig. 1. Penetration of sodium chloride into 0·5 per cent. agar gels containing 0·005*N* silver nitrate. The initial concentrations of sodium chloride are indicated.

From the data obtained experimentally by von Fürth and Bubanović these authors conclude that the influence of concentration on the march of diffusion can be expressed by the simple relation

$$d = mt^{\frac{1}{2}}c^n,$$

where d is the distance penetrated,
t „ „ time,
c „ „ concentration,
and m and n are constants.

In their experiments with sodium chloride they obtained $m = 41{\cdot}96$ and $n = 0{\cdot}12$, t being measured in days, d in millimetres and c in normalities.

Put into words this equation states that the march of diffusion is proportional to the square root of the time and to some fractional power of the concentration. The results recorded in Tables I to III show that the march of diffusion is approximately proportional to the square root of the time. This conclusion has also been reached in regard to penetration of acids, alkalies and salts into gels by Coleman [1888], Chabry [1888] and Voigtländer [1889]. How near this approximation is may be seen from the following tables, which show that the march of diffusion is generally approximately proportional to the square root of the time in the case of any particular gel and any particular concentration of penetrating salt. At the beginning of the diffusion the ratio $P/\sqrt{t}$ is constantly lower, and towards the bottom of a tube generally higher than the approximately constant value for the greater part of the experimental period. The greatest divergences appear when the concentration of the silver nitrate in the gel approaches that of the penetrating salt.

If now we return to von Fürth and Bubanović's equation $P = mt^{\frac{1}{2}}c^n$, where the distance penetrated is denoted by P and the initial concentration of the penetrating salt by c, we have

$$\frac{P}{\sqrt{t}} = mc^n,$$

or

$$\log\frac{P}{\sqrt{t}} = \log m + n \log c,$$

whence if $\log P/\sqrt{t}$ is plotted against $\log c$ a straight line should be obtained.

Table IV. 0·5 per cent. agar gel containing 0·005N $AgNO_3$. (For data see Table I.) P represents penetration in mm. and t the time in hours.

1·2N NaCl		N NaCl		0·5N NaCl		0·2N NaCl		0·1N NaCl		0·05N NaCl	
P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$
13·8	8·08	13·5	7·90	12·4	7·28	10·6	6·23	9·1	5·36	7·9	4·65
38	8·43	37·2	8·25	34·1	7·56	30	6·55	26·2	5·81	22	4·88
44·5	8·47	43·8	8·34	40	7·62	35	6·66	31	5·91	25·8	4·92
56	8·42	54·7	8·13	50·2	7·55	44·1	6·63	38·6	5·80	32·2	4·84
				64·5	7·71	55·1	6·59	48·1	5·75	40·1	4·79

Table V. 2 per cent. agar containing 0·1*N* $AgNO_3$.
(For data see Table II.)

5*N* NaCl		2*N* NaCl		*N* NaCl		0·5*N* NaCl		0·2*N* NaCl		0·1*N* NaCl	
P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$
10·5	6·41	7·9	4·94	6·3	4·05	4·8	3·15	2·4	1·63	1·0	0·690
14·5	6·51	11·2	5·09	9·1	4·20	7·4	3·45	4·1	1·94	5·0	0·825
39·2	8·04	25·8	5·31	21·3	4·39	17·1	3·54	10·2	2·06	5·5	1·008
45	8·30	29	5·36	24·5	4·54	19·4	3·68	12·1	2·21	7	1·002
58·5	7·65	37·5	5·40	31·5	4·54	25	3·61	15	2·14	8	0·950
		45·5	5·39	38·2	4·53	30·5	3·62	18	2·14	8·2	0·840
		53·5	5·48	44·5	4·51	35	3·59	25	2·56		

Table VI. 4 per cent. agar containing 0·05*N* $AgNO_3$.
(For data see Table III.)

5*N* NaCl		2*N* NaCl		*N* NaCl		0·5*N* NaCl		0·2*N* NaCl		0·1*N* NaCl	
P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$	P	$\frac{P}{\sqrt{t}}$
13·5	7·67	10	5·79	8·2	4·87	6·5	3·94	2	1·24	1·3	0·821
19·0	8·27	13·2	5·81	11·4	5·09	8·8	3·98	7	3·20	2·5	1·16
39	7·94	29·2	5·96	2·5	5·12	21	4·31	15·6	3·21	8·5	1·75
43	7·88	33	6·05	28·5	5·24	23·5	4·33	17·5	3·23	10	1·85
52·6	7·54	42·2	6·05	37	5·31	30	4·32	22·2	3·20	16·2	1·92
		64	6·54	53	5·42	42·5	4·35	30·5	3·13	18·4	1·89

In Table VII are given the average values of $P/\sqrt{t}$ and the corresponding values of c in normalities, and Fig. 2 shows the curves obtained when values of log $P/\sqrt{t}$ are taken as ordinates and values of log c as abscissae. It will be observed that they are certainly not straight lines, and accordingly von Fürth and Bubanović's equation does not hold. A reference to von Fürth and Bubanović's results discloses that these workers only used a very restricted range of concentrations of the penetrating solutions namely from N to $N/4$. This corresponds in the curves in Fig. 2 to the portions between the vertical lines *A* and *B*. Even within this very restricted range the curves are only very approximate straight lines when the concentration of silver nitrate in a gel is low. The statement of von Fürth and Bubanović that their law is of wide applicability certainly cannot be accepted.

Table VII.

0·5 per cent. agar containing 0·005*N* $AgNO_3$		2 per cent. agar containing 0·1*N* $AgNO_3$		4 per cent. agar containing 0·05*N* $AgNO_3$	
$\frac{P}{\sqrt{t}}$	c	$\frac{P}{\sqrt{t}}$	c	$\frac{P}{\sqrt{t}}$	c
8·35	1·5	7·38	5	7·83	5
8·15	1	5·28	2	6·05	2
7·54	0·5	4·39	1	5·20	1
6·55	0·2	3·52	0·5	4·23	0·5
5·73	0·1	2·10	0·2	2·92	0·2
4·82	0·05	0·886	0·1	1·61	0·1

The results of a large number of experiments are similar to those recorded above. They indicate that the law connecting the march of diffusion with the square root of the time can be extended with a certain degree of approximation and within certain limits to the case of penetration of an electrolyte into a gel containing a substance with which the penetrating substance reacts. Von Fürth and Bubanović's further extension of this law to include the concentration of the penetrating electrolyte is not of the wide applicability claimed for it, and only holds over very limited ranges of concentration and is then only a crude approximation.

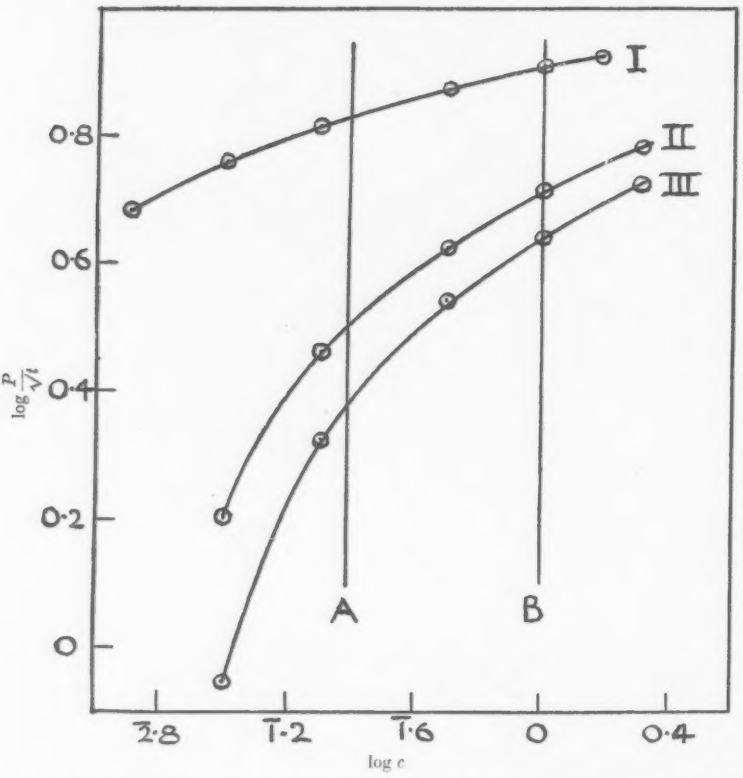

Fig. 2. Curves showing the relation between $\log P/\sqrt{t}$ and $\log c$ in the case of three agar-agar gels. I: 0·5 per cent. agar containing 0·005N $AgNO_3$; II: 2 per cent. agar containing 0·1N $AgNO_3$; III: 4 per cent. agar containing 0·05N $AgNO_3$. The vertical lines A and B indicate the limits of external concentrations employed in the experiments of von Fürth and Bubanović.

B. *Influence of concentration of silver nitrate in the gel.*

Although it is tacitly assumed by von Fürth and Bubanović that the concentration of silver nitrate in the gel is without influence on the march of diffusion, this is far from being the case. Tables VIII—X show some results representative of a great number which show clearly that increasing the concentration of silver nitrate in the gel reduces the rate at which the sodium chloride penetrates it. The results given in Tables VIII and X are also shown graphically in Fig. 3.

Table VIII. Penetration of sodium chloride of initially normal concentration into gels of 1 per cent. agar containing various concentrations of silver nitrate.

N $AgNO_3$		0·5*N* $AgNO_3$		0·2*N* $AgNO_3$		0·1*N* $AgNO_3$		0·05*N* $AgNO_3$	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
None		2·17	3	2·22	5	2·25	6	2·28	6·5
,,		4·55	5·1	4·58	7·6	4·60	9·4	4·62	10
,,		23·32	12·9	23·4	18·5	23·4	22	23·4	23·8
,,		28·90	14	29·0	21	29·0	25	29·0	27
,,		47·93	18·3	48·0	27	48·0	32	48·0	34·5
,,		70·9	21·5	70·9	32·5	70·9	39·5	70·9	42
,,		95·4	25	95·4	37	95·4	45·4	95·4	50

Similar results have been obtained with these concentrations of silver nitrate in the case of gels of 1, 2, 3 and 4 per cent. agar and of concentrations of sodium chloride ranging from 5*N* to 0·1*N*. But the same results are obtained when the range of silver nitrate concentrations is extended in the lower direction to the limiting concentration which is necessary for the silver chloride to be precipitated, as the following tables show.

Table IX. Penetration of 0·5*N* sodium chloride into gels of 0·5 per cent. agar containing various concentrations of silver nitrate.

0·05*N* $AgNO_3$		0·025*N* $AgNO_3$		0·005*N* $AgNO_3$	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
3·75	10·0	3·75	12	3·75	15
23·0	26·4	23·0	30	23·0	39
28·8	30·0	28·8	34	28·8	43·5
46·9	38·0	46·9	43	46·9	60
71·2	47·1	71·2	54		

Table X. Penetration of sodium chloride of initially normal concentration into 0·5 per cent. gels of agar containing various concentrations of silver nitrate.

0·005*N* $AgNO_3$		0·001*N* $AgNO_3$		0·0005*N* $AgNO_3$		0·0001*N* $AgNO_3$	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2·92	13·5	2·75	16·0	2·70	16·6	2·65	18·6
20·3	37·2	20·2	44·2	20·07	47·0	Precipitation of silver chloride not obvious after this time	
27·6	43·8	27·45	51·8	27·32	55		
44·25	54·7	44·1	66·0	44·1	75		
70	70	69·8	70				

These results show that in the lowest concentrations usable the concentration of silver nitrate in the gel influences the march of diffusion of sodium chloride through the gel. Thus von Fürth and Bubanović's comparison of the diffusion of chlorides and sulphates into gels containing unknown quantities of silver nitrate and barium nitrate as indicators is not legitimate.

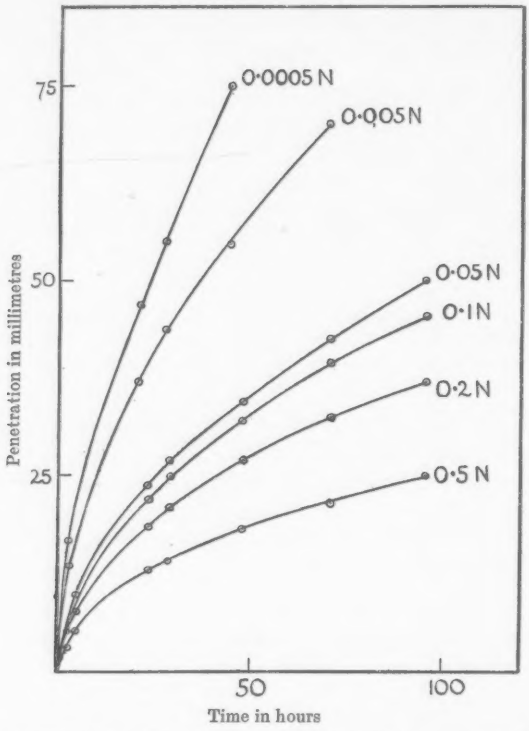

Fig. 3. Penetration of sodium chloride of initially normal concentration into gels containing different concentrations of silver nitrate as indicated.

C. *Influence of the concentration of the gel.*

No conclusive evidence could be obtained by the indicator method of any influence of the concentration of the gel on the march of diffusion. The results shown in Table XI are representative of a large number of series of experiments.

In this particular series the march of diffusion was slightly more rapid in the 0·5 per cent. agar than in more concentrated gels. In other series on the contrary it was slightly less rapid in the 0·5 per cent. gel than in others, while in yet other cases the penetration was quicker in gels of intermediate concentration than in gels of either the weakest or strongest concentrations. The

conditions are perhaps complicated by the action between the gel itself and the silver nitrate. If for instance a 2 per cent. agar gel combines with more silver nitrate than a 1 per cent. gel containing the same amount of silver nitrate, the concentration of silver salt able to combine with the chlorion of the sodium chloride may be less, and as has already been shown in the previous section of this paper the concentration of silver nitrate influences the rate of penetration of the sodium chloride into the gel. Loeb [1918] has recently brought forward evidence of the combination of silver nitrate with gelatin, and the colour change which sometimes takes place when silver nitrate is added to agar gels suggests that chemical compounds or adsorption compounds are produced. The method of preparing the gel probably influences the action between the agar and silver nitrate.

Table XI. Penetration of sodium chloride of initial concentration $2N$ into agar gels of various concentrations containing $0{\cdot}05N$ $AgNO_3$.

0·5 per cent. agar		1 per cent. agar		2 per cent. agar		3 per cent. agar		4 per cent. agar	
Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.	Time in hrs	Penet. in mm.
2·25	10	2·25	9·5	2·28	9·5	2·28	9·0	2·32	9·5
4·35	14	4·35	13·2	4·38	13·5	4·38	13	4·42	13
22·8	34·2	22·8	31	22·8	30·5	22·8	30·2	22·8	31·5
28·4	38·2	28·4	35	28·6	35	28·6	34	28·6	34
46·8	51	46·8	45	46·8	45	46·9	44	46·9	44·2

Under these circumstances the indicator method cannot be expected to yield very exact information as to the influence of the concentration of the gel on the rate of penetration of sodium chloride into the gel. This is a question which has already been the subject of a number of researches to which attention has been directed in the introduction to this paper. The divergent conclusions of Voigtländer working with chlorides diffusing in agar gels and Öholm on the diffusion of potassium chloride into gelatin are sufficient evidence of the unsettled state of this question.

D. *The influence of the concentration of silver nitrate in the gel considered in relation to the effect of concentration of the penetrating salt on the march of diffusion.*

In part A of the present section of this paper it has been shown that the rate of penetration of the gel by sodium chloride depends on the concentration of the penetrating salt, and in part B it is shown that it is also dependent on the concentration of silver nitrate. It is of importance to consider these two influences in conjunction, that is, how far the differences produced by varying the concentration of sodium chloride are affected by the concentration of silver nitrate in the gel.

In comparing the penetrating power of sodium chloride under different conditions it may be recalled that the penetration (P) is approximately proportional to the square root of the time (t) so that $P/\sqrt{t}$ is a constant for the

penetration of sodium chloride into any particular gel. It may be called the penetration factor. In the following tables are shown the values of $P/\sqrt{t}$ for a number of agar gels containing quantities of silver nitrate varying from N to $0{\cdot}05N$, with external solutions of sodium chloride of initial concentrations varying from $5N$ to $0{\cdot}1N$.

Table XII. Values of $P/\sqrt{t}$ for different concentrations of sodium chloride and silver nitrate.

1 per cent. agar.

	2N NaCl	N NaCl	0·5N NaCl	0·2N NaCl	0·1N NaCl
N $AgNO_3$	—	—	—	−2·54	−3·75
0·5 N „	4·802	2·64	1·44	−1·161	−2·64
0·2 N „	5·16	4·00	2·68	1·44	0·235
0·1 N „	5·58	4·65	3·56	2·56	1·37
0·05N „	6·64	4·97	4·21	2·93	1·93

2 per cent. agar.

	5N NaCl	2N NaCl	N NaCl	0·5N NaCl	0·2N NaCl	0·1N NaCl
N $AgNO_3$	3·825	—	—	—	−2·37	−4·06
0·5 N „	—	3·416	2·38	1·40	−1·327	−2·56
0·2 N „	—	4·50	3·59	2·65	1·35	0·00
0·1 N „	—	5·35	4·49	3·58	2·16	1·02
0·05N „	7·08	6·07	5·40	4·49	3·24	2·13

3 per cent. agar.

	5N NaCl	2N NaCl	N NaCl	0·5N NaCl	0·2N NaCl	0·1N NaCl
N $AgNO_3$	4·14	2·46	1·04	0	−2·44	−3·92
0·5 N „	5·16	3·71	2·47	1·51	−0·814	−2·59
0·2 N „	—	4·39	3·69	2·63	1·09	−1·00
0·1 N „	6·87	5·43	4·39	3·52	2·17	0·816
0·05N „	—	6·05	5·28	4·40	3·23	1·68

4 per cent. agar.

	5N NaCl	2N NaCl	N NaCl	0·5N NaCl	0·2N NaCl	0·1N NaCl
N $AgNO_3$	5·15	2·85	1·34	—	−2·09	−3·44
0·5 N „	6·91	3·70	2·54	1·565	−1·23	−2·64
0·2 N „	6·75	4·56	3·70	2·825	1·395	−0·982
0·1 N „	7·50	5·54	4·93	3·875	2·45	0·663
0·05N „	7·78	5·98	5·23	4·32	3·21	1·83

An inspection of the numbers in this table indicates that there is no suggestion of differences among them due to variation in the concentration of the gel, and differences in the corresponding numbers in the different tables are in strong probability best described as due to experimental error. The fairest way of considering them is to take the mean values of the corresponding numbers in the four sets of results. These mean values are shown in Table XIII.

In Table XIV are shown the values of the penetration factor obtained in two other series of experiments in which the concentration of silver nitrate

in the gel varied in one case between 0·05*N* and 0·005*N* and in the other between 0·005*N* and 0·0005*N*.

Table XIII. Values of $P/\sqrt{t}$ for different concentrations of sodium chloride and silver nitrate.

Mean values of the four sets of determinations recorded above.

	5*N* NaCl	2*N* NaCl	*N* NaCl	0·5*N* NaCl	0·2*N* NaCl	0·1*N* NaCl
N $AgNO_3$	4·37	2·65	1·19	0	−2·36	−3·79
0·5 *N* „	5·53	3·91	2·51	1·48	−1·13	−0·261
0·2 *N* „	6·75	4·65	3·745	2·70	1·32	−0·437
0·1 *N* „	7·18	5·475	4·615	3·63	2·335	0·967
0·05*N* „	7·43	6·185	5·22	4·355	3·15	1·89

Table XIV. Values of $P/\sqrt{t}$ for different concentrations of sodium chloride and silver nitrate.

(*a*) 0·5 per cent. agar.

	1·2*N* NaCl	0·5*N* NaCl
0·05 *N* $AgNO_3$	6·08	5·56
0·025*N* „	6·96	6·29
0·005*N* „	8·44	8·12

(*b*) 0·5 per cent. agar.

	1·2*N* NaCl	*N* NaCl	0·2*N* NaCl	0·1*N* NaCl	0·05*N* NaCl
0·005 *N* $AgNO_3$	8·44	8·27	6·63	5·82	4·86
0·001 *N* „	10·02	9·88	8·41	7·72	6·94
0·0005*N* „	10·71	9·96	9·17	8·56	7·84

From an examination of these tables certain facts are made clear. In the first place it is evident that the rate of penetration depends very much on the ratio of the concentrations of the penetrating sodium chloride and the silver nitrate in the gel. When the concentration of the latter is higher than that of the former the sodium chloride does not penetrate into the gel, but the silver nitrate on the contrary diffuses out into the sodium chloride solution, and if the experimental tubes remain unshaken, an advancing boundary of silver chloride is seen in contact with the pure sodium chloride solution. This is indicated in the tables by the minus sign. This being the case it might be expected that when the initial concentration of the penetrating liquid is only slightly higher than the concentration of silver nitrate in the gel penetration would only take place to a slight extent, for when withdrawal of sodium chloride by the silver nitrate has taken place to such an extent that its average concentration is reduced to that of silver nitrate in the unaltered part of the gel, no further penetration is possible. This is actually found to be the case. It is perfectly clear that such cases as these are not allowed for in the equation of von Fürth and Bubanović. It is also evident that with decrease of the silver nitrate content of the gel the relative difference in the penetration factor due to differences in concentration of the invading salt rapidly decreases. Thus with 0·1*N* silver nitrate the ratio of the penetration factors of 0·2*N* and

0·1N sodium chloride is 2·335 : 0·967. With 0·0005N silver nitrate this becomes 9·17 : 8·56. Earlier in this paper it has been pointed out that von Fürth and Bubanović's equation connecting time, distance, penetration and initial concentration of the penetrating salt cannot be accepted.

An approximate relation between the concentration, penetration and time becomes clear however on inspection of Fig. 4. Here the data given in Tables XIII and XIV (*b*) are represented graphically, the values of $P/\sqrt{t}$ being plotted against the logarithms of the concentrations. It will be observed that the relation between $P/\sqrt{t}$ and log c is approximately linear in the case

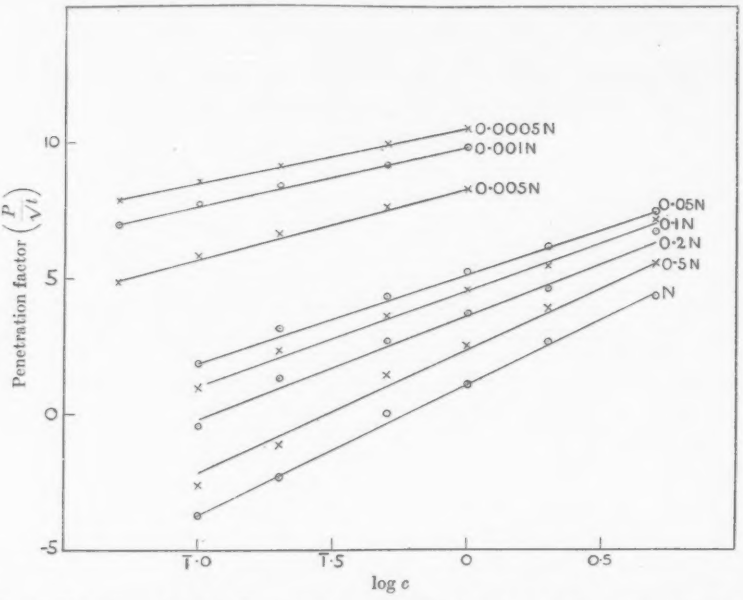

Fig. 4. The relation between the penetration factor ($P/\sqrt{t}$) and the initial concentration (c) of the penetrating salt in the case of a number of agar gels containing various concentrations of silver nitrate as indicated on the diagram.

of all the gels examined, these containing quantities of silver nitrate varying from N to 0·0005N and the initial concentrations of sodium chloride varying from 5N to 0·05N. This approximately linear relation between $P/\sqrt{t}$ and log c is thus of wide applicability. It must however only be regarded as a working approximation.

The equation connecting the penetration (that is the distance a definite concentration of sodium chloride has reached), time, and initial concentration of salt may therefore be written in the form

$$\frac{P}{\sqrt{t}} = k \log c + k',$$

where k and k' are constants for any particular gel, in which is involved the factor depending on the concentration of silver nitrate in the gel. It is almost unnecessary to point out that although the penetration factor is dependent on the initial concentration of the penetrating salt this has no bearing on the question whether the coefficient of diffusion is influenced by concentration. For the penetration means no more than the distance between the starting-point of diffusion and a layer of a certain definite, if low, concentration. If the coefficient of diffusion is uninfluenced by concentration, the penetration will obviously be greater in the case of an initially high concentration of penetrating salt than in one of low concentration.

Summary.

1. The penetration of sodium chloride into agar gels has been followed by the indicator method. When silver nitrate is present in the gel the entrance of sodium chloride into the gel is marked by the formation of silver chloride, and the progress of the chloride into the gel is indicated by the movement forward of the sharp line of demarcation between the silver chloride in the gel and the unprecipitated silver salt. The distance this line of demarcation has moved forward in any time is called the penetration. It marks the position of a definite concentration of chloride, namely, the saturation concentration of silver chloride.

2. The penetration of sodium chloride into agar gels containing silver nitrate is within wide limits proportional to the square root of the time. If P is the penetration in a time t, the value $P/\sqrt{t}$ is thus a constant for any particular gel and any particular concentration of penetrating salt, and may be called the *penetration factor*.

3. The rate of penetration is dependent upon the initial concentration of the penetrating salt, the higher the concentration of salt the more rapid the penetration.

4. The rate of penetration depends also on the concentration of the silver nitrate in the gel; the higher the concentration of silver nitrate, the lower the rate of penetration.

5. The concentration of the gel itself appears to exercise little influence on the rate of penetration, but owing to the probability of actions between the silver salt and the gel and hence doubt as to the real active concentration of silver salt in the gel, which, as stated in the preceding paragraph, influences the rate of penetration, the influence of concentration of the gel must be left an open question. In any case the influence cannot be great in gels containing between 1 and 4 per cent. agar.

6. The expression obtained by von Fürth and Bubanović to indicate the relation between the penetration, time, and concentration of penetrating salt only holds in very special cases and between very narrow limits, and is then only a crude approximation.

7. An empirical relation between these quantities of more general application is

$$\frac{P}{t^{\frac{1}{2}}} = k \log c + k',$$

where P is the penetration, t the time and c the initial concentration of the penetrating salt. The value of the constant k depends principally on the nature of the penetrating salt, and also, although to a slight extent, on the nature of the gel contents, while the value of k' depends principally on the concentration of silver nitrate in the gel.

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VIII. NOTE ON THE USE OF BUTYL ALCOHOL AS A SOLVENT FOR ANTHOCYANINS.

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In the purification and preparation of anthocyanins from flowers or fruits, there is need for a solvent, not miscible with water, which will extract the pigments from their solution in dilute acids. Amyl alcohol, the only solvent available so far, has the disadvantage that only mono- and rhamnoglucosides are soluble in it to any extent and even in their case the solubility is so small that as many as twenty extractions have been found necessary. The large bulk of solvent required to work up even small quantities of the colouring matter makes its use impracticable for the purification of the pigment in quantity. Willstätter and Zollinger in their work on the pigment of sloes, have therefore attempted to replace amyl alcohol by other solvents, without, however, finding a suitable one. (Phenol and cyclohexanol were the solvents tried.)

In the course of an investigation into the formation of the pigments in the grape vine, I observed that butyl alcohol removes all the anthocyanins (mono- and rhamnoglucosides as well as normal diglucosides) from their solution in dilute acids. The sugar-free pigments (anthocyanidins) are still more soluble in this solvent. On repeatedly washing the butyl alcoholic layer with fresh dilute acid, the non-glucoside pigments remain in the butyl alcohol, whilst the glucosidic anthocyanins pass again into the acid aqueous layer as oxonium salts. Butyl alcohol behaves therefore in this respect exactly like amyl alcohol.

As this observation promised to be useful for the purification of anthocyanins, I made some experiments with typical representatives of each type and compared quantitatively their solubility in butyl alcohol, taking their solution in amyl alcohol as a standard. In order to make the conditions comparable to those obtaining during their purification, the anthocyanin solutions were prepared by extracting the petals of flowers or skins of fruits with ethyl alcohol containing 1 per cent. hydrochloric acid and precipitating the filtered extracts with ether. After washing the pigments with ether and removing the latter with a current of air, they were dissolved in $N/2$ sulphuric acid and the solutions diluted to a uniform arbitrary tint. They were then divided into two parts, of which one was shaken out with an equal volume of

butyl alcohol and the other with amyl alcohol under identical conditions. The alcohols were previously saturated with the dilute acid employed, in order to keep the volume of the solutions constant. The relative strength of the first extract with each solvent was determined colorimetrically in a Hellige-Autenrieth's Colorimeter. The extractions were then continued until all but traces of the pigment was removed. The figures given under (1) in the following table refer to butyl alcohol only.

	Mono-glucosides			Rhamnoglucosides	
	Chrysanthemin	Oenin	Idaein	Prunicyanin	Violanin
1. Number of extractions	2–3	3–4	3–4	4–5	4–5
2. Solubility (amyl alcohol = 1)	4	4	10	16	25

The diglucosides examined were pelargonin, cyanin, and delphinin. They required from 7–8 extractions for the complete removal of the pigment. No comparative figures for their solubility in butyl alcohol can be given, as they are practically insoluble in amyl alcohol. It will be seen that even the less soluble anthocyanins possess a solubility in butyl alcohol four times as great as that in amyl alcohol, whilst that of the most soluble examined is twenty-five times as great. The unidentified anthocyanins of numerous flowers and fruits were, without exception, found to be soluble in butyl alcohol[1].

It would appear from these results that butyl alcohol, which is now a commercial product, would be of advantage in the preparation of mono- and rhamnoglucosides especially. The solubility of the diglucosides in the solvent, whilst not sufficient for purposes of preparation, is of such an order as to make it useful in those cases where their removal is essential, e.g. for the study of the colourless substances accompanying them in the plant.

I have successfully used butyl alcohol in the preparation, on a relatively small scale, of oenin (from grapes) and of idaein (from cranberries), the well-crystallised picrates of which may be easily obtained from the butyl alcohol extracts by the usual methods. Butyl alcohol may also replace ethyl alcohol and glacial acetic acid for the direct extraction of anthocyanins, especially from dried petals or leaves. In the latter case the pigment is only soluble in butyl alcohol when present as the oxonium salt of a mineral acid. This has the advantage that by a preliminary treatment of the dry powder with the solvent, such substances as chlorophyll, flavones, etc., are removed. The powder is then treated with butyl alcohol containing 2–3 per cent. hydrochloric acid, which extracts the anthocyanin as oxonium salt. From this solution the pigments are precipitated by light petroleum and purified by appropriate methods.

[1] It is interesting to note that the pigment of beetroot, which differs in many respects from anthocyanins, is insoluble in butyl alcohol.